The Impact of HIV and Antiretroviral Therapy on the Cardiovascular System of HIV-infected Children

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Statement of contributions

I confirm that the following people have contributed towards the data collection of work presented in this thesis.

All cardiovascular recordings were performed by Dorica Masaku, Dorothy Kavindele (UTH), Grace Mirembe, Florence Odongo and Priscilla Wavamunno (JCRC) and were analysed by myself. The immunophenotyping panels were run by Mpala Mwanza, Moffat Malisheni, Anglin Hamasuku (UTH), Patrick Olal and Lydia Nakiire (JCRC). All panels were re-analysed by myself to ensure consistency in the analysis. Professor Sarah Walker at the MRC Clinical Trials Unit at UCL has supervised and guided my statistical analysis.

I confirm that the rest of the work is my own, except where I have referenced the work of others.

Julia Kenny

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Abstract

Background

Cardiovascular disease is increased in HIV-infected adults but the underlying aetiology is incompletely understood. Pre-clinical changes in cardiovascular structure and arterial stiffness in HIV-infected children are described in mainly middle to high-income countries with minimal longitudinal data available. Limited cross-sectional data is available from Africa in settings where 90% of HIV-infected children live.

Methods

ART-naïve and ART-experienced (on d4T+3TC+NNRTI for >2years, virologically suppressed at enrolment) HIV-infected children underwent serial assessment within the CHAPAS-3 trial (evaluating d4T vs ZDV vs ABC-based first line ART in Zambia/Uganda): extensive cardiovascular assessment of structure (carotid intimal medial thickness [cIMT] and arterial stiffness (pulse wave velocity [PWV]). Additionally markers of inflammation, disordered thrombogenesis, vascular damage and immune activation were measured. Age-matched HIV-uninfected controls had a single assessment. Baseline differences between ART-naïve/experienced children vs controls, and longitudinal changes in HIV-infected children were assessed.

Results

In 208 ART-naïve children with median age 2.9y (IQR 1.7–4.4), median CD4% 18% (11-23) and 209 HIV-uninfected controls median age 3.0y (2.1–4.1), mean(sd) cIMT was 0.46(0.04) v 0.44(0.04) mm respectively ($p<0.001$); PWV was 5.85(0.8) vs 5.67(0.74)m/sec respectively ($p=0.05$). Among 74 ART-experienced children on ART for a mean of 3.7y with a median age of 6.9y (5.9–8.50), median CD4% 33% (27-39) and 75 uninfected controls with median age 6.7y (5.6-8.6), the mean(sd) cIMT was 0.46(0.05) vs 0.45(0.04)mm respectively ($p=0.09$); PWV was 5.63(0.61) vs 5.69(0.68)m/s respectively ($p=0.57$). In ART naïve children IMT and PWV significantly decreased from baseline (ART initiation) to week 96 mean(sd) cIMT -0.02(0.04)mm ($p<0.001$), PWV -0.37(0.82)m/s ($p<0.001$). In contrast whereas cIMT had significantly reduced by mean -0.2(0.06)mm ($p=0.01$) at week 96 in the ART experienced group PWV increased by 0.34(0.62)m/s ($p<0.001$). There was no

evidence that the changes in IMT or PWV over 96 weeks differed by randomisation ART in either ART naïve or ART experienced children ($p \geq 0.27$). Significant differences in a panel of 19 biomarkers and immunophenotyping (markers of activation and proliferation) were demonstrated between HIV infected ART naïve children and healthy age matched HIV uninfected children. No significant relationship between any of the biomarkers, immunophenotyping markers and IMT or PWV was demonstrated.

Conclusion

In this large study of arterial structural and stiffness in HIV-infected children in Africa, ART-naïve HIV-infected children had significantly poorer IMT and PWV than age-matched controls, with significant improvement seen after 96 weeks of ART. After a mean 3.7 years on ART, HIV-infected children had cIMT and PWV comparable to uninfected age-matched controls. IMT continued to improve after a further 96 weeks on ART. These findings illustrate that ART can reverse some of the structural/stiffness changes caused by HIV, strengthening the argument for early diagnosis and treatment of HIV-infected infants.

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Glossary of terms and abbreviations

3TC	Lamivudine
ABC	Abacavir
AIDS	Acquired Immune Deficiency Syndrome
APC	Allophycocyanin
ART	Antiretroviral Therapy
BD	Becton Dickinson
BD	Twice daily
BMI	Body mass index
CCA	Common carotid artery
CD	Cluster of Differentiation
CDC	Centre for Disease Control
CHAPAS	The Children with HIV in Africa Pharmacokinetics and Adherence/Acceptability of Simple Antiretroviral Regimens trial
CHER	The Children with HIV Early AntiRetroviral trial
CI	Confidence Interval
CIMT	Carotid Intima Media Thickness
CMV	Cytomegalovirus
CRF	Case Report Form
CVD	Cardiovascular disease
d4T	Stavudine
DAD	The Data Collection on Adverse Events of Anti-HIV Drugs
ddI	Didanosine
DICOM	Digital Images and Communication in Medicine

EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid,
EFV	Efavirenz
EID	Early infant diagnosis
ELISA	Enzyme-Linked Immunosorbent Assay
EMTCT	Elimination of mother to child transmission
FACS	Fluorescence-activated cell sorting
FBC	Full blood count
FDA	USA Food and Drug Administration
FDC	Fixed dose combination
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
Hb	Haemoglobin
HEU	HIV exposed, uninfected
HDL	High density lipoprotein
HIC	High Income Countries
HIV	Human Immunodeficiency Virus
hsCRP	High sensitivity C-reactive protein
ICA	Internal carotid artery
ICAM	InterCellular Adhesion Molecule
IL	InterLeukin
IL-1Ra	InterLeukin-1 Receptor Antagonist
IQR	Interquartile range
JCRC	Joint Clinical Research Centre, Kampala, Uganda

LMIC	Low to middle income countries
LPV/r	Lopinavir/ritonavir
MCP-1	Monocyte Chemotactic Protein-1
MFI	Median fluorescence intensity
MI	Myocardial Infarction
MRC-CTU	Medical Research Council Clinical Trials Unit at UCL
MTCT	Mother-To-Child Transmission
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NVP	Nevirapine
OD	Once daily
PBS	Phosphate-buffered saline
PE	Phyoerythrin
PENTA	Paediatric European Network for Treatment of AIDS
PerCP	Peridinin chlorophyll protein,
PI	Protease Inhibitor
PMTCT	Prevention of Mother to Child Transmission
PWV	Pulse Wave Velocity
RNA	Ribonucleic acid
SIV	Simian immunodeficiency virus
SMART	The “Strategies for Management of Anti-Retroviral Therapy” trial
SSC	Side scatter
START	The “Strategic Timing of AntiRetroviral Treatment” trial

TDF	Tenofovir
TNFα	Tumour Necrosis Factor alpha
TRECS	T-cell receptor excision circles
UNAIDS	The Joint United Nations Programme on HIV/AIDS
UTH	University Teaching Hospital, Lusaka
VCAM	Vascular cell adhesion molecule
VEGF	Vascular Endothelial Growth Factor
VL	Viral load
WHO	World Health Organisation
ZDV	Zidovudine

Chapter 1 Introduction and overview of thesis

The availability of antiretroviral therapy (ART) has created a new population of perinatally HIV-infected adolescents who are entering adulthood with an uncertain future ahead. Co-morbidities, including cardiovascular disease, are well described in horizontally HIV-infected adults and detrimental functional and structural arterial changes have been described in older children. No studies have looked at the extent of pre-clinical cardiovascular damage in very young HIV-infected children and minimal research has focused on children living in Africa, where over 90% of HIV-infected children live.

This research project was devised at a time when abacavir (ABC), a nucleoside reverse transcriptase inhibitor (NRTI), was associated in adults with cardiovascular damage, manifesting as an increased incidence of myocardial infarctions. The implications of these observations for children are uncertain. Optimising ART in childhood is a careful balance between efficacy, toxicity, cost and availability.

This thesis describes the cardiovascular health of a large cohort of young (aged 1 month – 13 years) children living in Zambia and Uganda. A total of 208 ART-naïve and 74 ART-experienced HIV-infected children were recruited to the cardiovascular sub study of a larger ART toxicity trial (CHAPAS 3; http://www.chapas3trial.org/) along with an equal number of age matched HIV-uninfected controls. A detailed cardiovascular assessment, including carotid intimal medial thickness (IMT) and pulse wave velocity (PWV), was performed at baseline and after 48 and 96 weeks of follow up.

The results show the influence of HIV-infection on vascular phenotype by comparing ART naïve HIV-infected children with controls. Longitudinal changes over 96 weeks are discussed along with the impact of three different ART regimens. Analysis of the cellular and circulating markers of inflammation, immune activation, and vascular damage measured have provided insight into potential mechanisms operating in HIV-infected young children living in Africa.

Chapter 2 Background

2.1 The changing nature of paediatric HIV-infection

In 1981 reports of an unexplained severe immunodeficiency in homosexual men living in San Francisco were published [1], shortly followed by reports of an acquired severe immunodeficiency in infants and children [2, 3]. Subsequently HIV was identified as the cause [4-6]. Without an effective treatment available it became clear that perinatally HIV-infected children had a very high mortality rate; twenty per cent died by 3 months of age, half died by 2 years of age and a majority had died by the age of 10 years [7, 8].

The discovery of the first antiretroviral drug, zidovudine (ZDV), brought hope that treating HIV was possible; however it soon became apparent that the virus could mutate and resistance quickly developed. New classes of antiretroviral drugs were developed and combined into dual and subsequently triple therapies for adults. The development of paediatric formulations lagged behind; the pharmacokinetic studies were expensive and intensive to run, limited liquid formulations were available for younger children and those available were unpalatable or required refrigeration. However more recently paediatric options have been expanded and where comprehensive paediatric HIV care is available, antiretroviral therapy (ART) has transformed the lives of perinatally HIV-infected children who are now surviving into adulthood [9, 10] and having (mostly uninfected) children of their own [11].

In 2016, in high income countries (HIC) mothers gaining access to comprehensive HIV care has resulted in mother to child transmission (MTCT) rates declining from 40% to under 1% [12]. In low to middle income countries (LMIC) and with the roll out of ART in the last decade, MTCT rates are higher but impressive reductions have been achieved; for example in a clinical trial setting in Botswana, perinatal transmission was 1.1% [13]. A shift to a programmatic, public health based approaches where all HIV-infected pregnant women commence and remain on ART for life is a major step towards making elimination of MTCT (EMTCT) an achievable goal.

For children for whom HIV preventive strategies were not available or were unsuccessful and who acquire HIV perinatally a healthy life is possible if ART can be

accessed. HIV as a chronic disease brings new challenges and the co-morbidities currently affecting HIV-infected adolescents living in HICs, whom have had access to ART for longer, need considering as the burden of co-morbidities become increasingly relevant globally [14]. Priorities in both HIC and LMIC include optimising ART provision, selecting effective yet affordable ART combinations whilst minimizing adverse effects and monitoring for co-morbidities which may not manifest clinically during childhood. Predictions are that the number of new paediatric infections will decline, but even with optimal scale up of ART in 2020, 1,940,000 children worldwide are expected to be living with HIV-infection [15]. Ensuring that this population of perinatally HIV-infected children reaches adulthood with future treatment options preserved and free from overt or silent co-morbidities is a challenge.

2.2 Epidemiology of HIV

2.2.1 Incidence and prevalence

The global HIV epidemic is thought to have peaked in 1996 when it was estimated that 3.5 million new infections occurred. Between 2001 and 2013 there has been a 58% decline in the number of new perinatally acquired HIV-infections; a success of a scale up of prevention of mother to child transmission (PMTCT) programmes. Despite these successes, 240,000 new paediatric infections still occurred in 2013. Globally an estimated 67% of pregnant women had access to HIV care and treatment. However disparities exist; in 10 out of the 21 of the UNAIDS/ World Health Organisation (WHO) Global Plan priority countries, MTCT rates remain over 15% [16]. By the end of 2013, 3.2 million children were HIV-infected, of whom 90% were living in Sub-Saharan Africa, Figure 2-1.

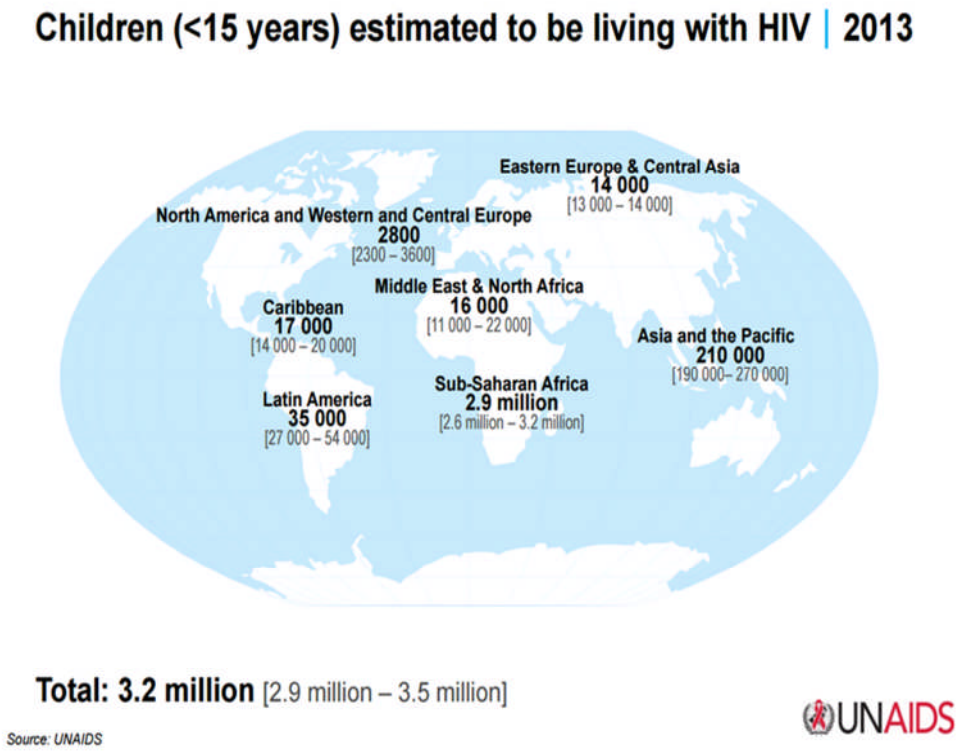


Figure 2-1. Estimated numbers of HIV-infected children by region in 2013.

Taken from UNAIDS Global Report 2014 [16]

2.2.2 Life expectancy without antiretroviral therapy

Untreated approximately 25% of HIV-infected children living in Europe exhibit rapid disease progression, dying by a year of age. After this age, approximately 5% of surviving children progress to AIDS each year, with 50% surviving to 10 years of age [17], Figure 2-2. A proportion of surviving ART naïve HIV-infected children have considerable morbidities including stunted growth, cardiomyopathies, bronchiectasis and neurocognitive impairment. However half of perinatally infected adolescents living in the UK were diagnosed after the age of 13 years and were asymptomatic at presentation [18].

Figure 2-2. Estimated time to progression to CDC clinical categories for all untreated children.

Kaplan-Meier life-table analysis of the outcome of children being cared for in 11 European centres [17].

HIV-infected, ART naïve children living in Africa have higher mortality rates. A large meta-analysis of mortality of infants born to mothers in one of 7 randomised African MTCT intervention trials (mothers received ART, no infant ART given) gave estimated cumulative mortality rates of 110 per 1000 live births by 12 months and 174 per 1000 live births at 24 months. At one year of age 35/5% of HIV-infected/uninfected infants had died; by two years this had increased to 53/8% respectively. Unsurprisingly maternal death, low maternal CD4 count and infant HIV-infection were all associated with increased infant mortality [7].

2.3 Anti-retroviral therapy

Until the mid-1990s ZDV monotherapy was the only treatment option. It wasn't very successful and resistance quickly developed. Dual therapies were introduced in 1996 and based on NRTI and NNRTI, often using drugs such as didanosine (ddI) and stavudine (d4T) which had undesirable side effects including the development of stigmatising lipodystrophy. Triple ART became available for children from 1997 and impressive data from the UK and Ireland based CHIPS cohort illustrated the dramatic reduction in hospital admissions, morbidity and mortality in HIV-infected children living in the UK after the introduction of triple ART - Figure 2-3, a trend seen across Europe and Northern America [19]. A study of over 3,500 HIV-infected children enrolled in the PACTG (now IMPAACT) 219 long-term follow up study in the USA. The death rate in 1994 was 7.2/100 patient years compared to 0.6/100 patient years in 2006 once ART was widely available. The age of death increased from 8.9 years in 1994 to 18.2 years in 2006, however mortality remained over 40 fold higher than HIV-uninfected age matched controls [20].

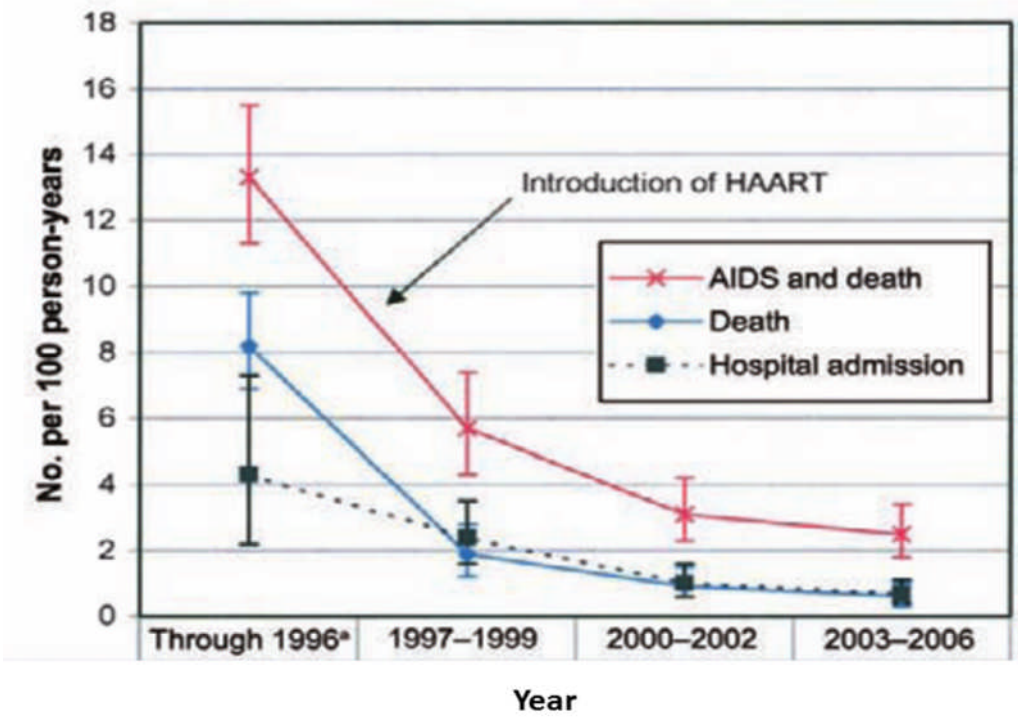


Figure 2-3. The dramatic reduction in hospital admissions, AIDS and death following the introduction of triple ART in children living in the UK and Ireland

Data from the CHIPS cohort covering the majority of HIV-infected children living in the UK and Ireland [19]

2.4 The global roll out of ART

Building on the successes of ART seen in HICs an international effort to provide ART across Africa began. The Global Fund to fight AIDS and the President's Emergency Plan for AIDS relief (PEPFAR) were amongst the first programmes to deliver ART to HIV-infected adults and subsequently children. Initially these were programmes dealing solely with HIV-infection, in larger urban centres and the success was impressive with substantial improvements in adult mortality for those who were able to access care [21, 22]. Fixed dose combinations (FDCs) were developed for adults that were easier to transport and store which simplified treatment delivery. The scale up of ART was made more feasible by the availability of low cost generic antiretroviral drugs produced initially by Cipla (Mumbai, India) and later by other companies.

Early in the global roll out of paediatric ART, syrup formulations, licensed in HICs, were used. Compared to tablets, syrups, were bulky to transport and store (often requiring refrigeration), complex to administer and expensive. Thus clinicians in some countries attempted to treat children by giving part of an adult FDC tablet. However unscored tablets were tricky to break, leading to unequal dosing and the ratio of some components, especially nevirapine (NVP) were unsuitable for young children, who require relative higher doses per kilogramme of body weight [23]. This lead to the development of the first paediatric FDC tablets, Triomune Baby and Junior, which combined stavudine (d4T), lamivudine (3TC) and nevirapine (NVP) with a higher ratio of NVP compared to adult formulations. The Children with HIV in Africa Pharmacokinetics and Adherence/Acceptability of Simple Antiretroviral regimens trial (CHAPAS-1) demonstrated good efficacy and tolerability of these formulations [24] and contributed to FDA approval in 2007. For many thousands of children in LMIC countries Triomune was lifesaving.

Unfortunately with time it became clear that d4T caused long-term changes in fat distribution (lipodystrophy and lipoatrophy) especially in peri/post-pubertal females, adults with higher CD4 counts and patients with a BMI of over 25 [25]; reassuringly pre-pubertal children appeared to be at less risk [26, 27]. Lipodystrophy was especially stigmatising and led to lower adherence to ART. Thus WHO 2010 guidelines recommended phasing out d4T restricting use only where alternative

nucleoside reverse transcriptase inhibitors (NRTIs) such as ZDV or abacavir (ABC) were not available or not tolerated.

Whilst the number of children on ART has tripled between 2008 and 2013, access to ART is not universal; in 2013 only 34% of children who were eligible, using current WHO treatment guidelines, received ART [16, 28]. Now there are over 25 antiretroviral drugs suitable for adult patients; but for children there are fewer formulations and dosing schedules are often based upon limited data. Not all antiretroviral drugs can be formulated into FDCs, and of the first line WHO recommended ART combinations for children under 12 years only one, ZDV/3TC/NVP is available as a FDC. Several gaps remain in available products for children before the 2013 WHO guidelines can be implemented in most LMIC. Optimising new paediatric friendly FDCs, which are harmonised to adult ART regimens, is a priority to ensure all HIV-infected children who need ART can access it. Simplifying treatment will help to decentralise HIV care and treatment to lower level health facilities integrating HIV care into broader primary health care.

Building upon adult experience, more paediatric appropriate FDCs are being developed; scored tablets allow doses to be easily tailored to the child's weight using the WHO weight band tables, dispersible tablets can be dissolved and given to infants and smaller children in suspension. New formulations of established antiretrovirals such as lopinavir/ritonavir (LPV/r) minitabs [29], efavirenz sprinkles, atazanavir powder and chewable raltegravir have or are being developed. There are expanded options for first, second and third line therapies including integrase inhibitors, CCR5 receptor antagonists and next generation non-nucleoside reverse transcriptase inhibitors, although these are expensive, prohibiting access for the majority of children in need of them.

2.5 The new generation of perinatally infected adults

With comprehensive HIV-care perinatally HIV-infected children survive childhood and enter adulthood [19]. In the UK/Ireland over 60% of children followed as part of the Collaborative HIV Paediatric Study (CHIPS) cohort are now aged 15 years and older, a reflection of very low numbers of newly infected children and improved survival of older children - Figure 2-4.

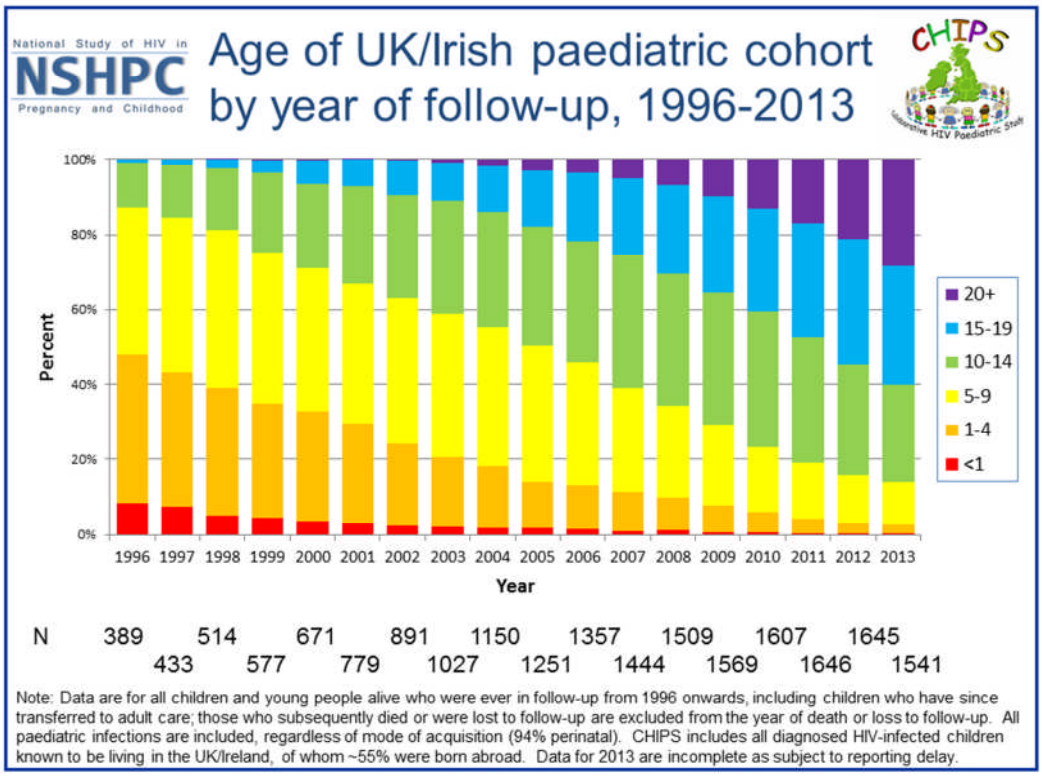


Figure 2-4. Change in the age distribution of UK/Irish paediatric HIV-infected cohort between 1996 and 2013.

An increasing proportion of HIV-infected children followed up in the Collaborative HIV Paediatric Study (CHIPS) in the UK and Ireland are now aged over 15 years and transiting to adult care. Reproduced with permission.

2.6 Causes of mortality in HIV-infected children and adolescents

In LMIC paediatric mortality rates remain high; in 2013 190,000 HIV-infected children were estimated to have died including 530 children a day from AIDS-related illnesses [16]. Causes of death include severe bacterial infections, cardiomyopathies and malignancies [30, 31]. In HIC mortality rates are falling and the causes of paediatric mortality are changing from end stage AIDS and AIDS related opportunistic infections to a notable increase in the proportion of non-communicable causes -Table 2-1, [20, 32]. Mortality in perinatally infected adolescents living in the UK/Ireland and Northern America who have transitioned into adult care settings is significantly higher than the general population, deaths mostly due to HIV-related conditions associated with virological failure and immune suppression [33, 34]. Within the UK this is mainly due to inadequate engagement with available care and ART adherence issues, as untreatable HIV in this population is thankfully very rare [33].

Table 2-1. Primary Cause of Death for HIV-infected Children living in the USA.

Grouped Cause of Death	Total n=298	Calendar Period 1994–1996 n = 152	1997–2000 n = 66	2001–2006 n = 80
AIDS-Defining Infection	92	55 (36.2%)	18 (27.2%)	19 (23.8%)
Mycobacterium avium complex	43	29 (19.0%)	9 (13.6%)	5 (6.3%)
Pneumocystis jirovecii pneumonia	27	14 (9.2%)	5 (7.6%)	8 (10.0%)
Cytomegalovirus	10	5 (3.3%)	2 (3.0%)	3 (3.8%)
Cryptosporidium	5	4 (2.6%)	1 (1.5%)	0
Cryptococcus/Toxoplasmosis	4	2 (1.3%)	0	2 (2.5%)
Invasive Candidiasis	3	1 (0.7%)	1 (1.5%)	1 (1.3%)
End-Stage AIDS	48	18 (11.8%)	11 (16.7%)	19 (23.8%)
Pneumonia	41	23 (15.1%)	13 (19.7%)	5 (6.3%)
Sepsis - bacterial or fungal	34	16 (10.5%)	6 (9.1%)	12 (15.0%)
Cardiomyopathy	20	11 (7.2%)	3 (4.5%)	6 (7.5%)
Central Nervous System Disease	19	12 (7.9%)	4 (6.1%)	3 (3.8%)
Malignancy	9	3 (2.0%)	3 (4.5%)	3 (3.8%)
Renal failure	7	1 (0.7%)	2 (3.0%)	4 (5.0%)
Stroke/cerebral Hemorrhage	5	1 (0.7%)	2 (3.0%)	2 (2.5%)
Hepatitis	4	2 (1.3%)	0	2 (2.5%)
Accidental	2	0	0	2 (2.5%)
Other/Unknown	17	9 (5.9%)	4 (6.1%)	4 (5.0%)

** = Test of trend in proportion of deaths due to each cause over calendar periods using Mantel-Haenszel Chi-Square test. For trends in Opportunistic Infection (OI)-related vs non-OI related deaths, the Mantel-Haenszel test p-value is 0.03.*

The children were part of the Paediatric AIDS Clinical Trials Group 219/219c long term follow up [20]

2.7 Paediatric HIV care in 2016

Without doubt in 2016, the availability of ART continues to be lifesaving. However HIV-infected children require ongoing access to comprehensive HIV care and considerable therapeutic, practical and psychosocial issues persist for HIV infected and affected children. These will be discussed in the following sections.

2.7.1 Making a diagnosis of HIV in infants

Without knowing the HIV-status of a child, appropriate care cannot be accessed but determining the HIV status of an infant in LMIC can be challenging. Due to the trans-placental passage of maternal antibody babies born to HIV-infected mothers are HIV-antibody positive regardless of HIV status. A definitive HIV diagnosis in infants under 18 months of age requires PCR confirmation. Ideally a mother is tested in the 1st trimester of pregnancy (with a repeat 3rd trimester test if she is at risk of seroconverting in pregnancy) and the baby tested after birth (by 2 months of age at the latest) and repeatedly until at least 6 weeks after the cessation of breastfeeding. Worryingly a recent study found that in babies who were being breastfed by mothers on ART, routine PCR assays to detect infant HIV DNA were insensitive. 22 weeks after cessation of breastfeeding were needed before a positive test result was obtained using standard PCR testing. Ultrasensitive testing confirmed the presence of HIV DNA before the routine PCR tests became positive. This suggests that maternal antiretrovirals can suppress HIV replication within an infant sufficiently to cause a false negative PCR result [35]. A single test at 6 weeks after cessation of breast feeding may be too early. Whilst early infant diagnosis (EID) is feasible in resource limited setting globally in 2013 only 42% of children exposed to HIV were tested by 2 months of age. Large geographical differences exist; for example whilst by 2 months of age 94% of infants in South Africa are tested, just 4% of infants in Chad and Nigeria have had a PCR performed [16, 36, 37]. Decentralisation of EID to rural health centres is integral to the success of an EID programme.

PCR testing requires technology that is usually only available at centralised large laboratories. PCR can be performed on dried blood spots which are simple to collect at a primary care level and can be easily transported for centralised testing. However long turnaround times getting the results back to the families, especially in remote areas, can be tricky. Innovative solutions such as using Global Package

Radio Service (GPRS) printers are promising in reducing turnaround times of returning results to the family [38, 39]. Point of care tests can provide decentralised EID providing same day results. Several technologies are under development although hurdles that need to be overcome include a limited number of samples tested per day, the sensitivity/specificity of each test and prohibitive costs [40, 41]. Targeting resources to the higher risk groups for testing is important, for example using precious resources to test children born to mothers who are in PMTCT programmes has a very low pick up rate of HIV-infection given the success of PMTCT interventions. Conversely testing all children admitted to a malnutrition unit or an acute medical word is likely to result in a far higher proportion of positive HIV results [42].

2.7.2 When to start antiretroviral therapy

The **C**hildren with **H**IV **E**arly Anti-**R**etroviral (CHER) trial provided unequivocal evidence that all children under the age of 2 years should be commenced on ART as soon as possible once a diagnosis of HIV is made, to reduce HIV related morbidity and mortality [43]. CHER data are supported by similar findings from a large European infant cohort meta-analysis [44]. The WHO expanded this finding to recommend that all children under the age of 5 years commence treatment and some African countries, such as Uganda, opted for all children to start ART without measuring CD4 counts, a pragmatic way of reducing the barriers to accessing ART. In May 2015 The Strategic Timing of ART (START) trial interim findings were announced early at a press release [45] and published shortly after [46]. This global trial took asymptomatic HIV-infected adults with a CD4 count greater than 500 cells/mm^3 and randomised them to either immediate ART regardless of immunological or clinical status, or withholding ART until their CD4 count fell below 350 cells/mm^3. The trial was terminated early as interim results provided compelling evidence of the benefit of early ART. These results have major global implications; the updated WHO 2015 guidelines recommended immediate treatment for all, including children. Early treatment aims to optimise immune status, neurodevelopment, minimising development of viral reservoirs, resistance and limiting toxicities.

Young children respond well to ART, and this is related to the ability of the immature immune system to reconstitute [47]. There is mounting evidence that delaying ART until the CD4 count drops impairs the potential for immune recovery [48, 49] and earlier treatment may be associated with lowered viral reservoirs [50-53], with time it may be that those with smaller reservoirs are candidates for future curative strategies. The European (PENTA 2015, [54]), American (DHHD 2014, [55]) and 2013 WHO guidelines [28] for commencing treatment prior to the release of the updated WHO 2015 guidelines are compared in Table 2-2.

Table 2-2. Comparison of previous guidelines for the initiation of antiretroviral therapy prior to the updated 2015 WHO recommendation of immediate ART for everyone.

Where resources are limited guidance on whom to prioritise is provided based upon World Health Organisation (WHO), US Department of Health and Human Services (DHHS) and Paediatric European Network for the Treatment of AIDS (PENTA) guidelines for antiretroviral therapy (ART) initiation. Table adapted from [54]

	WHO 2013	DHHS 2014	PENTA 2015
< 1 year	**All**	**All**	**All**
1 - 3 years	**All** *Prioritize* *1-2 years* *WHO stage 3/4* *CD4 count ≤ 750 cells/ul* *CD4 percentage ≤ 25%*	**CD4 count < 1000 cells/ul** **CD4 percentage < 25%** **CDC category B/C** **HIV VL > 100,000 copies / ml** *Consider* *All*	**CD4 count ≤ 1000 cells/ul** **CD4 percentage ≤ 25%** **WHO stage 3/4** **CDC category B/C** *Consider* *All**
3 - 5 years	**All** *Prioritize* *WHO stage 3/4* *CD4 count ≤ 750 cells/ul* *CD4 percentage ≤ 25%*	**CD4 count < 750 cells/ul** **CD4 percentage < 25%** **CDC category B/C** **HIV VL > 100,000 copies / ml** *Consider* *All*	**CD4 count ≤ 750 cells/ul** **CD4 percentage ≤ 25%** **CDC category B/C** **WHO stage 3/4** *Consider* *HIV VL > 100,000 copies / ml*
> 5 years	**CD4 ≤ 500 cells/ul** *Prioritize* *WHO stage 3/4* *CD4 count ≤ 350 cells/ul*	**CD4 count < 500 cells/ul** **CDC category B/C** **HIV VL > 100,000 copies / ml** *Consider* *All*	**CD4 count ≤ 350 cells/ul** **WHO stage 3/4** **CDC category B/C** *Consider* *CD4 count ≤ 500 copies / ml* *HIV VL > 100,000 copies / ml*

*"All", all children irrespective of immunological status; CDC, Centres for Disease Control and Prevention; VL, viral load. * In children aged 1 - 3 years consider ART in all especially if VL > 100,000 copies / ml*

2.7.3 First line antiretroviral options

WHO, European and USA guidelines provide recommendations on which ART combinations should be commenced [28, 54, 55]. Standard first line therapy in all guidelines consists of a three drug regimen made up of two nucleoside/nucleotide reverse transcriptase inhibitors with either a boosted protease inhibitor or a non-nucleoside reverse transcriptase inhibitor. The current WHO guidelines are listed in Table 2-3.

Some evidence from Africa suggests that LPV/r has virological advantages over NVP in neonates exposed to NVP perinatally although concerns exist about the interpretation of the trial findings. Where possible infected infants exposed to NVP during failed PMTCT should be started on a boosted PI containing regimen as transmitted resistance can lead to failure of NVP containing regimen [56, 57]. However LPV/r syrup is unpalatable, interacts with tuberculosis treatment and until recently was only available in a liquid formulation that required refrigeration. In May 2015 the USA FDA tentatively approved LPV/r pellets (Cipla) for infants and children aged less than 3 years. Whilst this is progress results from CHAPAS 2 showed that whilst pharmacokinetic data were similar between syrups and pellets caregivers initially preferred pellets at 12 weeks however by 48 weeks, mainly due to the adverse taste of the pellets, syrups were preferred [29, 58].

Table 2-3. Preferred and alternative first-line regimens for children.

Age Group	Preferred first-line regimens	Alternative first-line regimens
Children < 3 years	ABC or ZDV + 3TC + LPV/r	ABC + 3TC + NVP ZDV + 3TC + NVP
Children 3 years to less than 10 years and adolescents <35kg	ABC + 3TC + EFV	ABC + 3TC + NVP ZDV + 3TC + EFV ZDV + 3TC + NVP TDF + 3TC (or FTC) + EFV TDF + 3TC (or FTC) + NVP
Adolescents (10-19 years) ≥ 35 kg	TDF + 3TC (or FTC) + EFV	ZDV + 3TC + EFV ZDV + 3TC + NVP TDF + 3TC (or FTC) + NVP ABC + 3TC + EFV (or NVP)

3TC, lamivudine; ABC, abacavir; EFV, efavirenz; FTC, emtricitabine; LPV/r, lopinavir/ritonavir; NVP, nevirapine; TDF, tenofovir; ZDV, zidovudine;

Adapted from the 2013 WHO Consolidated ARV Guidelines [28]

In young children ABC remains a preferred first line treatment. As will be described further in section 2.10.8 concerns have been raised about the potential adverse cardiovascular effects of ABC and cardiovascular disease in adults. Alternatives such as ZDV, which has been associated with anaemia, have previously been used with caution in countries with high prevalence of malaria and anaemia. Tenofovir (TDF) has recently been given FDA approval from 2 years of age however concerns over renal and bone toxicity remain [59, 60].

The CHAPAS 3 study was the first study to compare first line treatment using d4T with ZDV or ABC in an African setting. The trial will be described in more details in section 3.1 but briefly 478 children, three-quarters of whom were ART naïve, were randomised to either d4T, ZDV or ABC with 3TC and either NVP or EFV. Children in all three arms did equally well; only 6% had to change any of their initial regimens due to side effects and 1% had to switch to second line treatment for virological failure. No difference was demonstrated between groups in terms of adverse effects (including anaemia), hospitalisations or disease progression. The majority of patients maintained virological suppression at 48 and 96 weeks with no difference between arms [27] however a relationship between long term use of d4T in older children was associated with adverse changes in markers of clinical lipodystrophy [61].

2.7.4 How to simplify antiretroviral therapy

With children facing a lifetime on ART once started on ART, it is critical to simplify treatment, optimise adherence, minimise toxicities and limit the development of resistance. Simplifying treatment is the first step to improve adherence. Once daily ART is far more convenient and leads to improved adherence [62]. The once daily administration of ABC/3TC from aged 3 months has been shown in several trials to be safe and efficacious [27, 63, 64].

2.7.5 Once started can antiretroviral therapy ever be stopped?

The **P**aediatric **E**uropean **N**etwork for the **T**reatment of **A**IDS (PENTA) 11 study investigated the effect of planned treatment interruption in 109 children and found, in a small group of carefully monitored children, this strategy was safe [65, 66]. This was in stark contrast to the findings from The **S**trategies for **M**anagement of **A**nti**R**etroviral **T**herapy (SMART) study. This was a large treatment interruption trial

that recruited 5472 adults, with a CD4 count of greater than 350cells/mm3 (the then threshold for commencing ART) and randomised patients to either continuous or CD4 count guided (treatment interruption) ART. The trial was terminated early when it was demonstrated that the treatment interruption arm had significantly increased incidence of opportunistic disease or death from any cause [67].

Within the paediatric population controversy remains as to whether ART can be safely interrupted once started. The long term follow up of the children recruited in to the CHER study showed that the infants whom received immediate ART for 96 weeks tolerated later treatment interruptions well, with 32% remaining off ART by the end of the 6 year trial. The CHER investigators concluded that treatment interruption after intensive time limited primary treatment was well tolerated and different to interrupting treatment in chronic HIV-infection, possibly reflecting a decreased HIV reservoir or reflecting young infants ability to reconstitute their CD4 cells, related to their increased thymic output [68].

The BREATHER trial has shown that in a highly selected group of children and adolescents short cycle therapy is non inferior to continuous ART. This trial took 199 8 – 24 year olds from across the world who were stable on first line efavirenz based ART and randomised half to take two consecutive days off ART each week. Young people in the trial randomised to the SCT arm enjoyed the freedom of not having to take ART at the weekends and reported that after a period of adjustment their quality of life improved [69].

2.8 Anti-retroviral therapy in 2016 and beyond

The evidence to start ART early is strong and it is without doubt that ART is lifesaving. There are concerns that periods of viraemia will replenish HIV reservoirs, increase immune activation and in the adolescent and adult population, increase the risk of transmission. However there are also concerns about the cumulative life time ART exposure in HIV-infected individuals. The choice of paediatric ART needs careful consideration to ensure that the delicate balance between cost, formulation and side effects is addressed to ensure success in mortality is not at the cost of increased long term toxicities and co-morbidities. Ongoing pharmacovigilance is important and often difficult to coordinate in resource limited countries [70].

2.9 Emerging complications

Before the availability of effective ART, opportunistic infections, end stage AIDS and HIV-related malignancies were the main causes of mortality in HIV-infected adults; the majority of adults progressed to end stage AIDS within 2-15 years of primary infection [71]. In 2016 if ART is commenced with a CD4+ count greater than 350 cells/mm^3, non-smoking horizontally HIV-infected adults whom subsequently achieve immune reconstitution (CD4+ counts above 500 cells/mm^3) and maintain virological suppression, are thought to have a mortality approaching that of the general population. Conversely a CD4 count less than 350 cells/mm^3 when ART is commenced may reduce life expectancy by 20 years [72-74]. However these figures may be overestimates as they exclude “out of care patients” and a survivorship bias for older patients who survived in the pre-ART era exists. Non-HIV related mortality by age remains higher than in the general population; the reasons for this are multi-factorial and yet to be fully determined. The leading causes of morbidity and mortality among HIV-infected adults on ART now include cardiovascular, pulmonary and renal disease [75].

2.10 Mechanisms implicated in cardiovascular dysfunction and disease in HIV-infected adults

Based upon mainly adult studies multiple mechanisms have been proposed to explain the increased risk of CVD in HIV-infected patients. Whilst the strongest evidence exists for inflammation (more pronounced in patients with detectable viraemia) and excessive immune activation (more pronounced in patients with virological suppression) contributing to accelerated atherosclerosis, no one clear mechanism has emerged. Among men with low coronary artery calcium scores HIV infection was associated with an increased prevalence of non-calcified coronary plaque independent of traditional cardiovascular risk factors. This finding adds weight to the argument that non-traditional pathways and more than one mechanism are implicated in the increased risk of CVD in HIV infected adults [76].

Potential mechanisms unique to HIV

a) Inflammation; related to the consequences of viraemia

b) Immune activation despite viral suppression or low level viral replication

c) Accelerated atherosclerosis

d) Endothelial dysfunction

e) Altered haemostasis

Risk factors

a) Viraemia
b) Advanced immune suppression
c) Co-infections
d) Exposure to certain antiretroviral medication especially older protease inhibitors and a controversial role of ABC

2.10.1 Inflammation; related to the consequences of viraemia

Current thinking places inflammation as a key component of the increased CVD risk in HIV-infected individuals.

Much of the initial data on the role of inflammation in HIV came from the SMART (Strategies for Management of Antiretroviral Therapy) trial. The SMART study demonstrated that uncontrolled viraemia itself can raise the risk of cardiac disease and cardiovascular risk is decreased in patients with sustained virological suppression. In the treatment interruption arm uncontrolled viraemia accompanied by elevated inflammatory indices was thought to be driving the increased CVD risk [77]. This prompted vigorous studies of inflammation as a possible mediator of CVD in treated and untreated HIV-infection [78].

HIV has pro-inflammatory actions as shown by increased expression of several inflammatory markers, such as, high-sensitivity CRP (hsCRP) [79-81] and IL-6 [78, 80] and the expression of endothelial adhesion molecules such as VCAM-1 [82]. Higher levels of such markers are associated with both AIDS related and non-AIDS related morbidity and mortality [83]. Increased progression of carotid IMT was associated with higher levels of hsCRP [84]. Viral suppression though ART usually improves these pro-inflammatory parameters [85, 86] and suggests an inflammatory set point. An impressive finding, that a single IL-6 and d-dimer can predict 10 year morbidity and mortality [87] unfortunately was not repeated in other studies

suggesting that risk can be modified through external factors [88]. However despite early ART persistent abnormalities in inflammatory markers remain suggesting there are other mechanisms at play [89].

The role of inflammation and risk of CVD is not unique to HIV-infected adults. In the JUPITER study, HIV uninfected adults with high levels of CRP but LDL-C levels below the threshold for lipid lowering agents were commenced on a statin with anti-inflammatory actions. Results showed that the patients in the arm randomised to statin therapy had a lower incidence of primary cardiovascular disease [90]. When a similar study, SATURN-HIV was carried out in a high risk HIV-infected population no reduction in cardiovascular events were seen in the patients randomised to statins [91]. Additionally the benefits in lowering of LDL-C levels were also not seen. Although the dose of rosuvastatin used in SATURN-HIV was half that used in JUPITER due to the interactions with PI use, even patients on PIs did not have the lipid changes expected. 48 weeks of rosuvastatin treatment reduced significantly several markers of inflammation including CD38+HLA-DR+ and lymphocyte and monocyte activation in ART-treated subjects[92]. These results taken together suggest that unique mechanisms are operating in HIV-infected individuals.

2.10.2 Immune activation despite viral suppression or low level viral replication

Early animal models suggested that immune activation had a role in the pathogenesis of AIDS. Silvestri et al demonstrated that in Sooty Mangabeys simian immunodeficiency virus (SIV) infection leads to high levels of viral replication without the development of AIDS and with a normal lifespan. In contrast Rhesus macaques develop AIDS. The difference being that the Sooty Mangabeys have minimal immune activation whilst widespread immune activation in the rhesus macaques is implicated in the development of AIDS [93].

The finding that adults who fail to reconstitute their CD4+ T cells whilst taking ART have a higher risk of cardiovascular disease may be explained by T cell activation causing persistent pro-atherogenic inflammation despite viral load suppression [94]. Early ART reduces the risk of CVD, delaying ART leads to persistently abnormal immune activation and a greater HIV reservoir [95].

2.10.3 Accelerated atherosclerosis

Atherosclerosis plays an important role in the pathophysiology of cardiovascular disease. Development of atherosclerosis is a result of complex interactions between intrinsic and extrinsic risk factors, both modifiable (such as type 2 diabetes mellitus, hypertension, and dyslipidaemia) and non-modifiable (such as genetic susceptibility). Coronary angiography studies show that asymptomatic HIV-infected adults have a doubling of risk for non-calcified, vulnerable plaques which are at increased risk for rupture compared to controls matched by Framlington risk scores [96, 97]. Mounting evidence supports HIV associated inflammation and immune activation driving atherosclerosis [98, 99] with contributions from both cellular and humoral immunity [100-102].

To understand how HIV affects the development and progression of atherosclerosis it is important to understand the underlying pathophysiology.

2.10.4 Endothelial dysfunction

The endothelium is a single layer of cells that provides the interface between the intra and extra vascular components. The layer is metabolically active and regulates haemostasis, cellular adhesion, vascular growth and coagulation partially through the production, expression and modulation of adhesion molecules and pro-inflammatory cytokines including TNFa, IL-6, sVCAM-1 and MCP-1. Endothelial dysfunction can be thought of simply as an imbalance between vasodilation and vasoconstriction substances produced by (or acting on) the endothelium [103]. Endothelial dysfunction is seen early in the atherosclerotic process and leads to reduced vasodilation, increased inflammation, impaired coagulation and fibrinolysis contributing to plaque initiation and progression. In HIV-infected patients endothelial dysfunction is well recognised and has been associated with both HIV-infection and antiretroviral therapy [104-107]. HIV-infection itself is associated with a more pronounced adhesion of leukocytes to endothelial cells [104] and high levels of the endothelial markers (ICAM-1, VCAM-1 and E-selectin) have been described in HIV-infected patients [105].

Fatty streaks / atheromata development

Within areas of endothelial dysfunction the first changes are the development of arterial fatty streaks, an accumulation of lipid-laden macrophages and T-cells beneath the endothelium [108]. Fatty streaks may regress or progress to atheromata. Atheromata are asymmetrical focal thickenings of the intima, the inner layer of the artery. Atheromata comprise a core of foam cells and extra cellular lipid droplets surrounded by a cap of smooth muscle cells and a collagen rich matrix [109].

Infiltration of atheromata and vessel walls

As illustrated in Figure 2-5 low density lipoprotein *(*LDL) infiltrates the arterial wall and is modified by oxidative and enzymatic reactions. The modified LDL particles induce expression of the leukocyte adhesion molecules VCAM-1 and ICAM-1 which attract the accumulation of activated inflammatory cells; these produce inflammatory cytokines [110-114]. Leukocytes then roll along the wall of inflamed vessels before adhering, infiltrating the atheromata and transmigrating. Monocytes are attracted to the endothelium via MCP-1, attach via ICAM-1 and VCAM-1 and become activated macrophages. Higher levels of sCD163 suggestive of monocyte activation are seen without an increase in more traditional measures of inflammation such as CRP [115]. Activated macrophages transform into foam cells after taking up large quantities of oxidized LDL. Further T cell activation amplifies the inflammatory response via production of mediators such as IFN-γ contributing to a pro-thrombotic local environment, more amenable to a build-up of plaques with lipid laden foam cells and a thin necrotic surface.

Plaque rupture or endothelial erosion

As illustrated in Figure 2-6 antigen presenting cells within the atherosclerotic plaque trigger a predominantly Th1 T cell response characterised by secretion of IFN-γ and further macrophage activation. T cell factors and mediators from the activated macrophages reduce plaque stability and release proteases that degrade the extracellular matrix. Activated macrophages may also produce pro-thrombotic and pro-coagulant factors that directly precipitate the formation of thrombus at the site of plaque rupture. Regulatory T cells modulate the atherogenic process by secreting anti-inflammatory cytokines whilst foam cells set up a cycle of chronic inflammation and plaque rupture. The activation of the atherosclerotic plaque precipitates

ischaemia and infarction either as a cause of plaque rupture or endothelial erosion, if this affects coronary blood flow myocardial infarction ensues.

Figure 2-5. The initial stages in the atherosclerotic process.

1. *Infiltration of the arterial wall by LDL*
2. *LDL is modified by oxidative and enzymatic reactions.*
3. *Modified LDL particles induce expression of VCAM-1 and ICAM-1*
4. *Monocytes migrate into the vessel wall and differentiate into macrophages*
5. *Macrophages take up oxidized LDL and transform into foam cells*
6. *Activated T-cells produce pro-inflammatory cytokines that further amplify the inflammatory response*

Adapted from [158]

Figure 2-6. The role of T-cells in the pathogenesis of arterial plaque development.

1. *Within the atheromata antigen presenting cells trigger an inflammatory response casusing furhter activation of macrophages*

2. *Pro-inflammatory cytokines reduce the stability of plaque and release proteases that degrade the extracellular matrix.*

3. *Activated macrophages also produce prothrombotic and procoagulant factors that directly precipitate the formation of thrombus at the site of plaque rupture.*

4. *Regulatory T cells modulate the atherogenic process by secreting anti-inflammatory cytokines.*

Adapted from [158]

2.10.5 Altered haemostasis

Evidence for the role of altered coagulation in the development of end-organ disease, including myocardial infarction during HIV-infection, is accumulating [78]. Abnormalities predisposing to a hypercoagulable state have been reported in both ART naïve and experienced HIV-infected adults including presence of the lupus anticoagulant [116], deficiencies of protein C and protein S [117, 118], heparin cofactor II [119], anti-thrombin [120] and increased levels of von Willebrand factor [121, 122] although many of these abnormalities correlate with the severity of HIV-associated immunosuppression and with the presence of co-infections [123].

2.10.6 Co-infections

Infections other than HIV can act as a trigger or mediators of the inflammatory response in atherosclerosis and both HIV-infected children and adults are at increased risk of co-infections [124]. In a study of Ugandan HIV-infected children attending an outpatient clinic 92/140 (66%) and 57/73 (78%) ART experienced and ART naive children had detectable Epstein Barr virus (EBV) DNA levels. Mean EBV DNA levels were lower in ART experienced children and tended to be inversely associated with ART duration [125].

Co-infections such as CMV or hepatitis C have also been shown to have adverse effects on circulating levels of pro-inflammatory cytokines [126]. For example Italian adults with CMV/HIV coinfection had increased severe non–AIDS-defining events/non–AIDS-related death, especially with cardiovascular and cerebrovascular events, independently of other prognostic factors [127]. A cohort of HIV/CMV co-infected adults living in the USA, despite effective ART had elevated CD4 and CD8 T cell activation [128] and detectable CMV is associated with higher HIV DNA levels after early commencement of ART which may affect the stability of the HIV DNA reservoir [129].

2.10.7 ART related toxicities

Without doubt uncontrolled viraemia is a risk factor for CVD and the benefits of ART are clear. Optimising ART regimens to reduce toxicities that have been implicated in the pathophysiology of CVD is important. As summarised in Table 2-4 some antiretroviral drugs have direct effects on cardiovascular disease or indirectly though metabolic derangements such as insulin resistance and dyslipidaemia may contribute towards increased CVD [130].

2.10.7.1 Dyslipidaemia

Historically the protease inhibitor (PI) class has been considered to have a major impact on pro-atherogenic serum lipid elevations [131, 132], The D:A:D team demonstrated that cumulative exposure to PIs contributed to an increased rate of MI thought to be partially (but not fully) explained by PI associated dyslipidaemia [133] a finding seen in several other cohorts [134, 135].

However HIV-infected ART naïve children and adults also have abnormal lipid profiles so HIV infection itself appears to also cause a dyslipidaemia. A particular dyslipidaemia has been described in HIV-infected ART naive adults; lower levels of high-density lipoprotein (HDL) cholesterol that inversely correlates with HIV RNA levels. This is thought to be due to HIV mediated interruption of the reverse cholesterol transport pathway through which cholesterol is cleared from the peripheral tissues [136].

ART naïve and experienced HIV-infected children have been shown to have adverse lipid profiles [137] (including high non-HDL-C, LDL-C triglycerides and low HDL-C) with less favourable profiles seen with current PI use [138-140].

The role of different subclasses of lipids in the pathophysiology of atherosclerosis is becoming increasingly understood. A key initiating set in the process of atherosclerosis is thought to be the sub endothelial retention of apolipoprotein B containing lipoproteins. These retained lipoproteins then initiate local responses including a chronic T-cell and macrophage driven inflammatory response. This is then thought to drive the development of atherosclerotic lesions. Ongoing work looking at therapeutic manipulation of this pathway aims to further reduce the risk of

atherosclerosis [141]. Recently post prandial hypertriglyceridemia has become established as a risk factor for the development of atherosclerotic lesions. Overproduction and/or decreased catabolism of triglyceride rich lipoproteins predispose to hypertriglyceridemia especially in those with a genetic predisposition or who are obese. Post prandial accumulation of triglyceride rich lipoproteins promotes the retention of remnant particles in the arterial wall. Remnant particles contain up to 40 times the amount of cholesterol compared with LDL. The larger size of remnant particles cannot cross the endothelium as efficiently as smaller LDL particles and predispose to accelerated atherosclerosis and increased risk of cardiovascular disease [142]. To date no work has examined these pathways in HIV-infected patients.

Table 2-4. Antiretroviral therapy and impact of individual drugs on lipid and glucose metabolism and coronary artery disease.

Class of ART	Antiretroviral	Effects on lipids*	Effects on glucose •	Impact on coronary artery disease
Nucleos(t)ide reverse transcriptase inhibitors	Abacavir	TC↑ LDL↑	No effect	Potential increase in MI following recent exposure (see section 2.x)
	Emtricitabine	Neutral effect	No effect	No known association
	Lamivudine	Neutral effect	No effect	No known association
	Stavudine	Dyslipidaemia +	Insulin resistance +	No known association
	Tenofovir	TC↓ LDL↓	No effect	No known association
	Zidovudine	TC↑ LDL↑	Insulin resistance +	No known association
Non-nucleoside reverse transcriptase inhibitors	Efavirenz	TC↑ LDL↑	No effect	No known association
	Etravirine	Neutral effect	No known effect	Insufficient patients exposed
	Nevirapine	HDL↑	No known effect	No known association
	Rilpivirine	Neutral effect	No known effect	Insufficient patients exposed
Protease inhibitors	Amprenavir + Ritonavir	Dyslipidaemia ++	Insulin resistance +	Cumulative exposure independently increased risk for MI
	Atazanavir + Ritonavir	Dyslipidaemia +	Insulin resistance +	No known association
	Darunavir + Ritonavir	Dyslipidaemia +	Insulin resistance +	Insufficient patients exposed
	Indinavir	Dyslipidaemia +	Insulin resistance +++	Controversial results
	Lopinavir + Ritonavir	Dyslipidaemia +++	Insulin resistance +++	Cumulative exposure independently increased risk for MI
	Nelfinavir	Dyslipidaemia +	Insulin resistance +	No known association
	Saquinavir	Dyslipidaemia +	Insulin resistance +	No known association
	Tipranavir + Ritonavir	Dyslipidaemia +	Insulin resistance +	Insufficient patients exposed
Integrase inhibitors	Elvitegravir / cobicistat	Neutral effect	No effect	Insufficient patients exposed
	Raltegravir	Neutral effect	No effect	Insufficient patients exposed
Entry inhibitors	Maraviroc	Neutral effect	No effect	Insufficient patients exposed

** Dyslipidaemia defined as increased total cholesterol (TC), low density lipoprotein cholesterol (LDL), triglycerides and decreased high-density lipoprotein cholesterol (HDL); • + weak effect; ++ moderate effect ; +++ important effect; ↑ increase ; ↓ decrease, MI myocardial infarction.*

Adapted from [174]

2.10.7.2 Lipodystrophy

The use of ddI, d4T and EFV are associated with lipodystrophy, especially in adolescents and adults. Lipodystrophy in younger children does occur but less frequently [143-146]. In the entire CHAPAS 3 cohort (478 children) only two children, both of whom were older and had been on d4T for a mean of 3.9 years at enrolment, developed lipodystrophy.

Expanding or altering the distribution of visceral fat may promote the development of atherosclerosis; the visceral fat deposits are metabolically active and may promote an inflammatory reaction. Epicardial fat, given its close proximity to the coronary vessels may influence atherosclerosis due to the pro-inflammatory cytokines released. Recent work has shown that epicardial fat is higher in HIV-infected adult males and positively associated with duration of ART, especially ZDV and correlated with increased coronary calcium and subclinical coronary atherosclerosis [147].

2.10.8 Abacavir and cardiovascular disease – an ongoing debate

In 2008 the unexpected results of the D:A:D cohort created a storm of controversy [129]. The D:A:D cohort was a prospective observational cohort study of 33,347 patients followed in total for 367,559 person-years during which 517 patients had a myocardial infarction (MI) however, a lack of HIV-negative controls meant this could not be compared to the expected age-related incidence. Investigators found a 16% increased relative risk of acute MI with every year of exposure to ART. Initially this was linked to the use of PIs but after adjusting for use of NRTI class and lipids an attenuated risk of 10% risk per year of ART exposure was found. The initial hypothesis, that ZDV and d4T were implicated, was rejected after further analysis suggested that exposure to ABC within the last 6 months was associated with a 1.90 relative risk of acute MI and to a lesser extent cumulative use of ABC also was associated with an increased MI risk [84, 130, 131]

Following this multiple retrospective analyses have been carried out looking for an ABC signal. An independent analysis of the patients in the continuous arm of the SMART dataset also gave a signal that ABC, was associated with an increased risk of cardiovascular disease [132] and combined with the D:A:D results led to the USA

Department of Health and Human Services ART guidelines reclassifying ABC from a preferred first-line agent to an alternate agent in November 2008.

Conflicting reports over the relationship of cardiovascular disease with ABC continue to be published. These are summarised in Table 2-5 and no clear answer has emerged, with opposing opinions being obtained when even the same data set is analysed [133, 134]. It is important to highlight that most conclusions have been drawn from cohorts or trials that were not specifically designed to assess ABC cardiotoxicity and given the low cardiovascular event rates most cohorts are underpowered. Furthermore, differences in study designs, populations, and scope of data collection have meant no studies have been able to specifically address the risk of MI following recent ABC exposure.

A major confounder in the cohort studies is channelling bias; individuals at higher risk of metabolic disease, lipodystrophy or cardiovascular disease may have been preferentially treated with ABC after previous studies had shown a favourable change in lipid profile when PIs were changed to ABC [135-138]. Similarly impaired renal function is a cardiovascular risk factor in HIV-infected individuals [139, 140]; individuals with known renal dysfunction may have been preferentially prescribed ABC as the commonest prescribed NRTI alternative, tenofovir, poses a risk of renal toxicity [141].

The studies showing an association between ABC and cardiovascular events tended to include patients who were virologically suppressed, and those that did not report an association (generally the meta-analyses of RCTs) generally included ART-naive subjects. It may be argued that in ART-naive patients, the overall benefits of achieving virological suppression and limiting ongoing inflammation with ART may outweigh any potential negative effects of ABC, or alternatively the trials did not have sufficient follow up time for any risk to manifest.

To overcome the large numbers and long term follow up needed to specifically address the risk of CVD with ABC, smaller studies have looked at indirect biomarkers of cardiovascular risk and pre-clinical markers of vascular dysfunction. No adverse changes in patients on ABC have been seen in a large number of studies [142-153]. A small trial of 40 adult patients randomised to either ABC or TDF containing regimens revealed a small transient increase in E-selectin and sVCAM-1

at week 4 but long term there were no significant differences in a panel of biomarkers that also included hsCRP and d-dimer [154]. A small study of 35 adults switching to an ABC containing regimen showed increased levels of various inflammatory markers including hsCRP compared to adults switching to a non-ABC regimen [156]. Finally a study of high risk male adults revealed moderate improvements in cardiac biomarkers and arterial stiffness when switched from an ABC containing regimen to a TDF containing regimen [157].

Table 2-5. Summary of cohort analyses investigating associations between the use of abacavir and cardiovascular disease.

Total patients	RR CV event with ABC use (95% CI)	Comments
Increased risk of cardiovascular disease with abacavir use		
Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D) [148-150]		
33,347 HIV+ (178,835 patient yrs) Update 367,559 patient yrs	1.9 (1.47 : 2.45) 1.98 (1.70 : 2.29)	Potential for confounders and remember benefit of ART. Update - a strong association between current ABC use and MI risk remains, channelling bias unlikely
Independent analysis of SMART data [151]		
2752 HIV+	4.3 (1.4 : 13.0)	ABC may cause vascular inflammation which may precipitate a CVD event
Nested case control – Quebec (QPHID) [152]		
7053 HIV+ 27681 HIV-	1.79 (1.16 : 2.76)	An association with EFV and LPV/r use also noted. No data on smoking / HIV disease status.
*****Cohort study - USA Veterans Study [153]***		
10931 HIV+	1.48 (1.08:2.04)	Recent ABC exposure associated with increased CVD.
Danish prospective nationwide cohort study [154]		
2952 HIV+	2.0 (1.1 : 3.64)	Association confirmed but confounders not fully accounted for.
North Carolina Medicaid (comparator cohort study) [155]		
3481 HIV +	2.05 (0.72 : 5.86)	Need either large prospective RCT or larger observational study
RCT – treatment simplification – STEAL study group[156]		
357 HIV+	2.79 (1.76 : 4.43)	A larger study with more CV events needed for a more definitive result
Australian retrospective case–control study [157]		
68 HIV+ with CVD 136 HIV+ no CVD	OR:2.10, p=0.03	PI therapy, HIV viral load and duration of known HIV-infection were not predictive:
A systematic review and meta-analysis of studies [158]		
23 studies***	1.09 (1.02:1.16)	Inconsistent findings, duration of ABC treatment important.
NA-ACCORD [159]		
14,785 HIV+ no ABC 1,948 HIV+ on ABC	1.71 (1.11 : 2.64) Adjusted 1.34 (0.96 :1.88)	Increased MI risk with recent ABC use but reduced statistical significance after adjusting for RF
Swiss HIV Cohort Study [160]		
11,856 HIV+	2.06 (1.43 : 2.98)	ABC cumulative increase in risk of a CVD event.
Kaiser Permanente, California [161]		
8154 HIV+	2.2 (1.4 : 3.5)	Increased risk with ABC not explained by confounders.

No relationship between cardiovascular disease and abacavir use		
GSK analysis: A pooled analysis of 52 manufacturer trials including 12 RCTs [162]		
9502 HIV+ on ABC 4672 HIV+ not on ABC	0.81(0.38:1.75)	Need further data, very few MI overall
A pooled analysis from 5 clinical trials (ALLRT / ALTG) [163]		
5056 HIV+ starting ART, 17,404 patient years	0.6 (0.3 : 1.4)	More important to assess classic CV risk factors
Meta-analysis of RCTs [164]		
28 RCTs 9233 HIV+	0.95(0.62:1.44)	No evidence ABC increases risk of major CV event
*****a retrospective observational cohort study using Veterans dataset (VACCR) [165]***		
19424 HIV+	1.18 (0.92 : 1.5)	Increased use ABC with chronic kidney disease.
Nested case control – analysis plan specifically looking at ABC and risk of MI within the French Hospital Database) [135]		
74,958 HIV+	2.01 (1.11 : 3.64)	Increased risk of ABC confined to those who used cocaine / iv drugs.
The HEAT study a RCT comparing ABC v TDF [166]		
688 HIV+	Not given	No increase in CVD in ABC group and all explained by other RF.
The US Food and Drug Administration (FDA) meta-analysis of RCTs [167]		
26 RCTs 9868 HIV+	Risk difference -0.008% (20.3%: 0.3%).	No association between ABC use and MI risk.
10 year post marketing surveillance in Japan [168, 169]		
1087 HIV+ (2453 patient years)	Not given	Japanese population – lower incidence of CVD, possible incomplete reporting or not assigning risk to ABC (industry sponsored)

ABC Abacavir; BMI body mass index; CAD coronary artery disease; CVD cardiovascular disease; ART antiretroviral therapy; HDL high density lipoprotein; HEAT study, the HIV Study with Epzicom And Truvada; MI myocardial infarction; PI protease inhibitor; pyrs patient years follow up, RCT randomized controlled trial.
SMART definition Major CVD – MI, stroke, surgery for CAD, cardiovascular mortality. Expanded CVD – major CHD + heart failure, peripheral vascular disease, CAD needing treatment, unwitnessed death.
Reporting CVD, defined as myocardial infarction (MI), ischaemic heart disease, cardiovascular and cerebrovascular events or coronary heart disease
*** These 2 have conflicting results yet same data set*
**** Of the 23 studies included in the meta-analysis 3 cross sectional, 2 case controlled, 16 cohort studies and 2 RCTs.*

2.10.9 Postulated mechanisms linking ABC and cardiovascular disease

Since the first reports of an interaction between ABC and CVD, extensive research to determine underlying the mechanisms through which ABC may be exerting an effect on cardiovascular disease has been undertaken.

Endothelial dysfunction

In virologically suppressed patients an independent association between ABC use and endothelial dysfunction was observed [170]. ABC has been shown to cause down regulation of endothelial nitric oxide synthetase and superoxide anion production which leads to leucocyte accumulation and endothelial dysfunction [171-173].

Pro-inflammatory actions

Proposed pro-inflammatory actions of ABC could contribute to the atherosclerotic process by increasing plaque instability and risk of rupture. Analysis of the SMART study found higher levels of hsCRP and IL-6 in patients on ABC [151], a small study of 10 patients commencing ABC showing significant increases in pro-atherogenic inflammatory biomarkers [174] and in pregnant women in Botswana significantly higher levels of 4 pro-inflammatory cytokines were seen in the arm randomized to ABC [175]. However other population-based studies or post hoc trial analyses which have measured pro-inflammatory biomarkers have failed to provide convincing evidence [176].

Dysregulation of gene expression

Recent work investigated changes in gene expression in subcutaneous adipose tissue biopsies using microarray in HIV-infected patients commencing ZDV, TDF or ABC containing ART and followed for up to 24 months. Results at all time points showed that patients receiving ABC displayed a markedly different pattern of gene expression within subcutaneous adipose tissue. The differences were particularly marked at month 6 but still evident at month 24. The ABC group showed enrichment of a number of pathways involved with cell processes and cell communication (adherence junction, focal adhesions, tight junctions) and environmental information processing (WNT signaling, Extra-cellular membrane (ECM) receptor interaction,

leukocyte trans endothelial migration, neuro-active ligand interactions, MAP-kinase signaling) highlighting some novel mechanisms by which ABC may interfere with the endothelial adhesion process and endothelial function in subcutaneous adipose tissue, particularly early in the treatment. In particular abnormalities in tight junction and adherence junction brought about by ABC use, if observed in other tissues, may be a contributing factor to cardiovascular events in persons already with some underlying risk for atherosclerosis and may require further study [177].

2.10.10 Cardiovascular disease in HIV-infected adults

Much research has investigated cardiovascular disease (CVD) in HIV-infected adults; reported rates of cardiovascular disease have, until recently, remained stubbornly high and the incomplete understanding of the pathophysiology has limited intervention strategies. Absolute risk of HIV-associated CVD varies depending on the population studied (differences between LMIC and HIC), access to ART and prevalence of other risk factors. Different patterns of cardiovascular disease have emerged and appear to be influenced by ART exposure.

ART naïve patients

Without ART advanced immunosuppression and opportunistic infections dominate leading to pericardial disease (often secondary to tuberculosis), dilated cardiomyopathies and pulmonary hypertension [178-181].

Recently commenced or stopped ART or intermittent ART

The SMART study demonstrated that intermittent CD4 cell count guided ART treatment was associated with an unacceptably high risk of cardiovascular events in patients in the treatment interruption arm [67].

ART experienced with virological suppression

With ART an increase in CVD, manifesting clinically as higher rates of myocardial infarction (MI), ischaemic heart disease (IHD), peripheral vascular disease, pulmonary hypertension and cerebrovascular disease (mainly stroke), has been reported [158, 182-185].

Table 2-6 summarises the main results of the larger cohort analyses of HIV-infected adult patients. A summary of the findings of the 10 largest analyses from North American and Europe are illustrated in Figure 2-7. Whilst a significant increased risk is seen in 9, the study designs were not ideal. These estimates are based upon retrospective cohort data and adjusting fully for risk factors, such as obesity, smoking and drug use, is not always possible. An assumption that HIV-infected and uninfected populations are broadly similar in terms of co-morbidities, socioeconomic and lifestyle factors is incorrect; HIV-infected adults aged over 45 years have a greater burden of co-morbidities compared to age matched controls [186, 187]. Additionally it is suggested that HIV-infected individuals have increased susceptibility to some risk factors; for example in a cohort of 17,996 HIV-infected, ART experienced, adults living in Europe and North America a two fold increase in mortality was seen in HIV-infected smokers compared to non-smokers (all-cause mortality rate 7.9 / 4.2 per 1000 person years for smokers / non-smokers) [188].

Freiberg et al [189] used the large Veterans Aging Cohort Study Virtual Cohort to carefully match HIV-infected to uninfected controls and found HIV-infection was associated with a 50% increased risk of acute MI beyond that explained by recognised risk factors. An excess risk (hazard ratio 1.39; 95% CI, 1.17 – 1.66) remained even in adults who had achieved virological suppression. Similarly using traditional cardiovascular risk prediction scores, developed on a population level, appears to underestimate the cardiovascular risk in HIV-infected adults [190].

At least two different underlying pathophysiological mechanisms are implicated in the development of a MI; primary (type 1) due to atherosclerotic plaque rupture and secondary (type 2) due to supply demand mismatch. Recent evidence suggests that type 2 may be more common in HIV [191]. Many of the studies presented within this chapter have not been able to differentiate MIs by type which confuses the interpretation into potential mechanisms by which HIV is influencing cardiovascular risk.

The final bar of Figure 2-7 presents study findings showing no increase in the incidence of MI above that of the general population [192]. Patients were Californians with medical insurance and with aggressive attention to and treatment of cardiovascular risk factors the investigators have shown it has been possible to reduce that rate from previously published higher rates from the same cohort [193, 194].

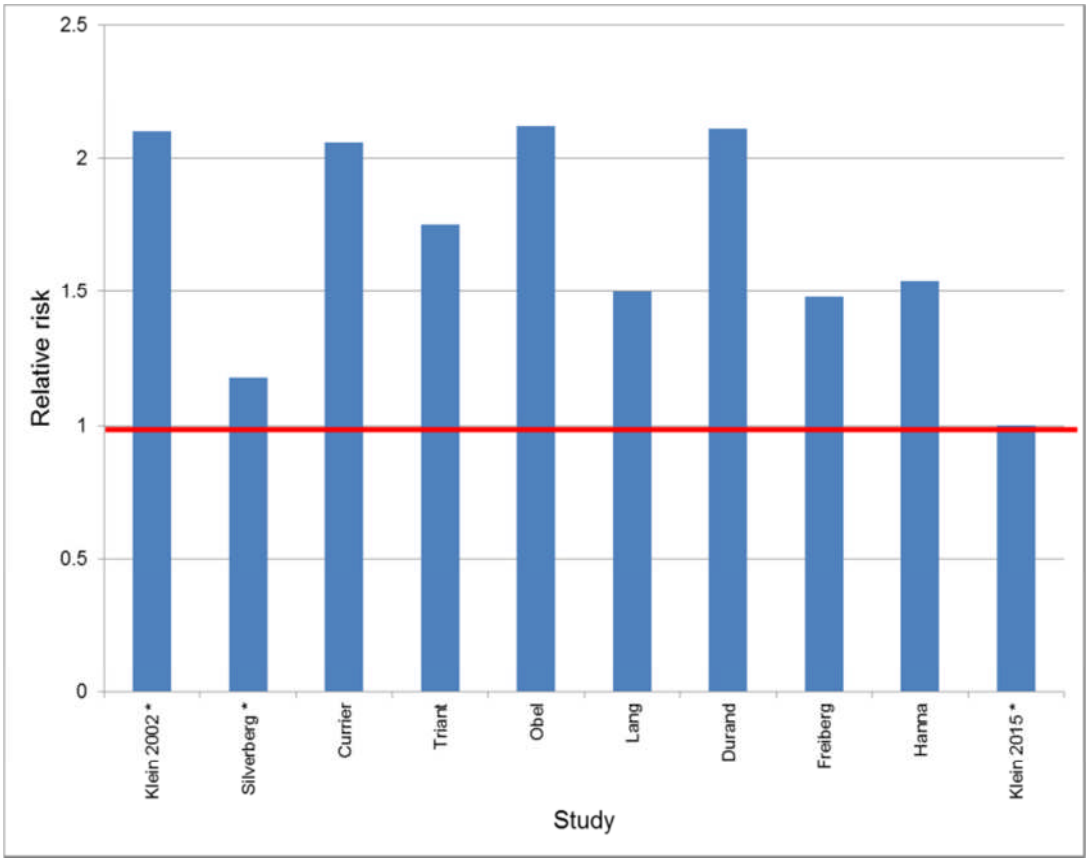


Figure 2-7. Relative risk of cardiovascular disease in HIV-infected adults compared to the general population.

*Based on 10 analyses of 8 cohorts. The 3 studies marked with a " * " are all based upon the same cohort – the Kaiser Permanente cohort in Northern California. The Silverberg results show the risk in patients with a CD4 > 500. The 2015 Klein results show that with intensive HIV care and aggressive management of cardiovascular risk factors it is possible to return the cardiac risk to background levels. Red horizontal line demonstrates background risk.*

[152, 189, 192-199]

Table 2-6. Summary of cohort studies reporting incidence of cardiovascular disease in HIV-infected adults

Population	Number / age of cohort / controls	Primary result	Ref.
Increased cardiovascular disease reported in HIV+ adults			
D:A:D	23,437 HIV+, no controls, median 39 yrs	Increased CVD with PI exposure and recent (but not cumulative) use of ABC and ddI. Limited evidence of accelerated CVD with increasing age.	[133, 149, 200, 201]
French Hospital Database	34,976 HIV+, no controls, mean 38yrs	Increased cardiovascular events seen with PI exposure.	[202]
Californian Medicaid	28,513 HIV+ 3.053,696 HIV-, age 18–75 yrs	CHD increased in HIV+ males <34 yrs and women <44yrs, RR 2.06 (p<0.001) of CVD if on ART and aged 18-33yrs. Effect not seen in older age bands	[195]
* Kaiser Permanente	22,081 HIV+, 230,069 HIV-, age 18 – 65 yrs	CVD increased in HIV+, no effect of PI or ART. CD4 >500cells/mm^3 protective, lower CD4 count increased risk of MI in HIV+	[193, 194]
American Partners Health care	3851 HIV+, 1,044,589 HIV–, Age 18-84 yrs	MI increased (RR 1.75, p<0.0001) in HIV+, especially in women. Increased co-morbidities seen in HIV+, unknown smoking status.	[196]
HIV Outpatient Study	6945 HIV+, no controls, median 42 yrs	Decreased CVD with increasing use of ART, relationship between PI use and MI (adjusted HR 6.5 95%CI (0.9 : 4.78)	[134, 203]
Veterans Aging Cohort Study Virtual Cohort	27,376 HIV+, 55,083 HIV-, median 49 yrs.	Increased MI in HIV+, HR 1.48 95%CI (1.27 : 1.72), even if virologically suppressed HR 1.39 95% CI (1.17 : 1.66)	[189, 204]
Danish National Hospital Registry	3953 HIV+, 373,856 HIV-, median 37yrs	HIV+ on ART have increased CVD: ARR 2.12 95%CI(1.62 : 2.76), no increase in the 8 years after ART initiation	[197]
New York City HIV Surveillance Registry	24,768 HIV+, 257,600 HIV-, mean 41 yrs	Both viraemic and virologically suppressed HIV+ have increased CVD until age 65 yrs RR1.54 (1.47 : 1.62).	[199]
Quebec dataset	7053 HIV+ 27681 HIV-, mean 40yrs	Increased MI in HIV+, RR 2.11 (95%CI 1.69 : 2.63), increased risk with ABC, EFV, LPV/r, no data on smoking status nor HIV clinical status.	[152]

No difference in overall cardiovascular risk between HIV+ and control population			
American Veterans Affairs Healthcare system	1st analysis: 36,766 2nd analysis: 41, 213 no controls age 35-55yrs	1st analysis: No increase in CVD with ART 2nd analysis a small increase in CVD; HR 1.05, 95%CI(1.05 : 1.06) if on ART, older and pre-existing vascular disease. Lower risk for African Americans HR 0.8 95% CI(0.69 : 0.98). Ongoing benefit of ART after 6 years.	[205, 206]
* Kaiser Permanente in Northern California	24,768 HIV+, 257,600 HIV- mean 41/40 yrs	With intensive HIV care decline in CVD. RR 1.0 (95% confidence interval, .7–1.4) in 2010–2011.Comment that may be underlying immune activation and subclinical changes not yet detected. In HIV+ evidence of similar/superior CVD risk profiles (these patients had ben intensively managed)	[192]

*ABC, abacavir; ART, anti-retroviral therapy; ARR, adjusted relative risk; CHD, coronary heart disease; CI, confidence interval; CVD, cardiovascular disease; DAD, data collection on adverse events of Anti-HIV drugs; ddI, didanosine; EFV, efavirenz; HR, hazard ratio; LPV/r, Lopinavir/ritonavir; MI, myocardial infarction; PI, protease inhibitor; RR, relative risk; *Kaiser Permanente cohort.*

2.10.11 Assessment of pre-clinical cardiovascular changes

The use of clinical end points to assess the risk of CVD is not ideal as the low incidence per year necessitates large patient numbers followed for long periods for sufficient power to detect statistically significant differences. An alternative strategy is to look at pre-clinical cardiovascular changes. Techniques that have been employed to examine pre-clinical changes include:

Intimal medial thickness (IMT)

IMT is measured by external ultrasound (also possible to measure invasively) and is the combined thickness of the tunica intima and tunica media, the innermost two layers of the wall of an artery. IMT will be described in greater detail in sections 3.2 and 3.3 but briefly carotid artery IMT can be used as a surrogate endpoint for evaluating the regression and/or progression of atherosclerotic cardiovascular disease. An IMT greater than 0.9-1mm is indicative of atherosclerosis and increased risk of cardiovascular disease.

Pulse wave velocity (PWV)

PWV, will be described in greater detail in sections 3.2 and 3.4 but briefly is a non-invasive method of measuring arterial stiffness. It has a strong correlation with cardiovascular events and all-cause mortality.

Computed Tomography (CT) angiography

CT angiography can be used to visualize atherosclerotic disease. It is more precise than ultrasound but requires the injection of contrast material, thus exposing the patient to a not insignificant dose of ionizing radiation, making its use in a paediatric research setting inappropriate.

Ankle brachial pressure index (ABPI)

ABPI is the ratio of the blood pressure in the lower legs to the blood pressure in the arms measured using a Doppler ultrasound probe. Abnormal results can be indicative of peripheral arterial disease.

Studies in HIV-infected adults using these techniques are summarised in Table 2-7. The majority of these studies show subtle but detrimental changes in HIV-infected

individuals compared to age matched controls. Some signals suggesting subtle differences between ART classes are seen although the implications are not always fully understood.

In summary, earlier epidemiological and cohort studies, albeit not perfect, suggest unique patterns of CVD in HIV-infected patients, with an increase in CVD at a younger age, in both men and women when compared to the general population. This excess risk cannot be explained by correction for traditional risk factors and it is clear that the established algorithms for identifying adults at high risk of CVD are not accurate in HIV infected adults. The lack of effect after the age of 65 years in some cohorts may be a reflection of a survival bias. Recent findings that CVD risk can be returned to the background population rate with intensive management of cardiovascular risk such as strict treatment of dyslipidaemia, hypertension and glycaemic control [83] are promising. However ongoing research needs to focus upon the complex pathophysiology of the increased cardiovascular risk in adults; hypotheses will be explored in the next section.

Table 2-7. Pre-clinical cardiovascular changes in HIV-infected adults

Study population	Number and age of cohort	Primary result	Ref.
Evidence of accelerated pre-clinical changes in HIV-infected adults			
Cross sectional study of FRAM and MESA study groups	433 HIV+, 5749 HIV- mean 49/61 yrs	HIV+ significantly greater IMT after adjusting for confounders. The risk conferred by HIV was similar to that of smoking.	[207]
San Francisco Study of the Consequences of PI Era (SCOPE) cohort	148 HIV+, 63 HIV-, mean 45 yrs	Increased and accelerated progression of IMT in HIV+, IMT changes proportional to classic risk factors and CD4<200 cells/mm^3 associated with an increased IMT. CMV specific T-cell responses independently associated with IMT.	[208] [209]
SMART – randomised CD4 guided treatment interruption	4831 HIV+ with no prior IHD	Over 11% asymptomatic HIV-infected adults had ECG changes of myocardial ischaemia. Traditional RF factors were the predominant determinants of risk. No clear association between class/duration of ART and asymptomatic IHD was noted.	[210]
Males recruited from Boston Clinics	78 HIV+ 32 HIV- Mean 47/45yrs	CT angiography used to assess coronary plaque – higher proportion of HIV+ had plaque (59% v 34%, p = 0.02), affecting more coronary segments (2.2 v 1.2 p = 0.03) and increased plaque volume (173 uL v 85uL, p = 0.02)	[96]
Beijing, China	82 HIV+ 43 HIV-	HIV is a RF for peripheral arterial disease, no adverse effects of NRTI / NNRTI	[211]
Ugandan cross sectional outpatient study	245 HIV+ median age 37 year (IQR 31-43)	18% had thickened IMT diagnostic of subclinical atherosclerosis (14% of ART nave, 24% of ART experienced)	[212]
START arterial elasticity sub-study – baseline results	337 of the START patients	Impaired small arterial elasticity with older age and among those with prior CVD, women and those of black race, also observed differences by global region	[213]
Multicenter AIDS cohort study	618 HIV+ 383 HIV- 40-70 yrs	Coronary artery plaque, especially non calcified plaque is more prevalent and extensive in HIV infected males independent of traditional RF for CVD,	[97]
Boston Medical Centre ID clinic	75 HIV+ 223 HIV-	Impaired FMD in HIV+, associated with current IV drug use and low α-high density lipoprotein triglyceride levels.	[214]
No evidence of pre-clinical changes in HIV-infected			
American multi-site study	44 HIV+ on PI 44HIV+ not on PI 44 HIV-	No statistically significant difference over 3 years in IMT progression between HIV-infected and un-infected adults. Higher homocysteine and LDL cholesterol predicted IMT progression	[215]
Washington University General Clinical Research Centre	50 HIV+ 50 HIV-	No difference in IMT progression between HIV+ stable on ART and HIV- adults. Insulin resistance a strong predictor of IMT.	[216]

CT ECG; FRAM, Fat redistribution and metabolic change in HIV-infection; IMT; MESA, multi-ethnic study of atherosclerosis;

2.11 Cardiovascular morbidity and mortality in HIV-infected children

Similar to the patterns seen within the adult HIV-infected population specific patterns of CVD are emerging in children with no/suboptimal ART exposure and with prolonged ART exposure. Of concern cardiac manifestations may remain undetected in childhood/adolescence and not manifest until early adulthood.

Antenatal factors

Antenatal factors can impact on later CVD risk. In some, but not all, cohorts it is reported that children born to HIV-infected mothers have a higher incidence of low birth weight and prematurity, especially if on a PI containing regimen or commencing ART late in pregnancy [217-219]. Prematurity and low birth weight are both known risk factors for the later development of cardio-metabolic disease [220, 221] microvasculature changes, elevated blood pressure and increased cIMT [222]. HIV-exposed uninfected (HEU) children exposed to antenatal ART have left ventricular structural changes and diastolic dysfunction, the long term significance of which is as yet undetermined [223, 224]. A need for longitudinal cardiac studies in HEU children assessing long-term cardiac risk is recommended to determine the implications of these findings.

ART naïve HIV-infected children

Similar to the findings in HIV-infected adults in the pre-ART era or when ART was commenced only in the late stages of immunosuppression left ventricular hypertrophy, dilated cardiomyopathy, congestive heart failure, severe dysrhythmias, cardiac tamponade, pulmonary hypertension and pericardial effusions in children and adolescents were common. Specific risk factors for the development of structural cardiac disease identified include no or short duration of ART, ZDV monotherapy and advanced AIDS [225-230].

ART experienced HIV-infected children

Since the introduction of effective ART the incidence of clinically overt CVD has fallen; for example the prevalence of cardiomyopathy fell from 44% to 3.7% with the introduction of ART [231], however an increased risk of cardiomyopathy remains

and ongoing ZDV exposure is implicated [232]. There is evidence that cardiac abnormalities where ART is available are mostly mild and asymptomatic in childhood [231, 233]. In HICs a worrying increase in the prevalence of cardiac risk factors are described in HIV-infected children; for example in one study 20% of a HIV-infected cohort of children were hypertensive, a finding of concern. The life-long cardiovascular risks associated with HIV-infection and its management mandate the need for closer monitoring and possibly treatment of elevated blood pressure in this population [234]. A significant proportion of HIV-infected adolescents living in the USA have been shown to have high coronary and aortic atherosclerotic risk scores, worse in adolescents on protease inhibitors and not taking tenofovir (although this may be a challenging bias associated with not putting adolescents with renal disease on tenofovir) [235].

As the complications of atherosclerosis (myocardial infarction, stroke and peripheral artery disease) occur later in life surrogate endpoints have been used for the detection of early signs and disease changes. Anatomical studies have shown that the atherosclerotic process starts in childhood, even in healthy HIV-uninfected children [236] and evidence is accumulating that this is accelerated in HIV-infected children and adolescents [237]. Non-invasive methods such as PWV, IMT and Flow Mediated Dilation (FMD) are well validated methods of assessing impaired vascular function and structure have been shown, in some studies, to be abnormal in HIV-infected young people, Table 2-8. However to date studies have been small, cross sectional and mostly conducted in middle to high income countries with a heterogeneous mix of ART exposure and regimens. The additional confounders of sedentary lifestyle, obesity and dyslipidaemia have been difficult to control for. To date only a single cross sectional study and no longitudinal studies have been conducted in an African setting [238].

Table 2-8. Summary of pre-clinical cardiovascular changes in HIV infected children and adolescents.

Population	Country	Mean age in years (range)	Parameters measured	Ref.
Differences between HIV+ and controls				
49 HIV + (34 on ART for median 96 months, 15 ART naïve, 27 PI based) 24 HIV– age /sex matched	France	13.5 (3.5:19) 12 (5:17)	IMT: No difference in IMT seen between groups (0.47 v 0.49mm) however reduced variation in CCA diameter between systole and diastole and reduced distensibility in HIV+, no difference between ART naïve and experienced. FMD: Reduced FMD in HIV+	[239]
100 HIV + (all on ART, for median 129 months, 48 PI based, 82 undetectable VL) 50 HIV -	Thailand	15.5 (12:20) 16.1 (12:19)	IMT: proximal and distal CCA and ICA, no difference between HIV+ and controls. In HIV+ higher IMT if on PI regimen (0.38 v 0.36mm) Echo:4 HIV+ had abnormal myocardial function – all 4 on PI based regimen.	[240]
83 HIV + (56 ART experienced, 48 on ART for median 5 years, 31 PI experienced, 23 on PI, 27 ART naive) 59 HIV– unmatched	UK	11.0 (5: 18) 12.2	IMT: Significantly increased IMT (0.6 v 0.47) seen in HIV + - age and PI exposure significant. FMD: Reduced FMD in controls compared to HIV+, also reduced with PI use PWV: Increased PWV (7.5 v 7 m/s) in HIV+ with an age effect seen, no difference between ART naïve and controls, no effect of PI	[241, 242]
83 HIV + (73 on ART for median 84 months, 37 on PI, 6 ART naïve) 83 HIV - age, sex and economic class matched	Brazil	10.8 (sd 2.6) 10.7 (sd (2.9)	IMT: Increased IMT (0.48 v 0.426) in HIV+, increased IMT associated with stavudine use (no PI effect), high CD8 and low CD4, lower IMT seen with higher TC and CD8 zenith.	[243]
150 HIV + (145 on ART, 115 VL undetectable) 150 HIV - age, sex and BMI matched	Spain	14.9 (3: 24) 14.7 (3:23)	IMT: thicker in HIV + v HIV – (0.434 v 0.424mm). HIV independently associated with increased IMT. Low CD4 associated with higher IMT. No relationship between IMT inflammation, immune activation or senescence.	[244, 245]

No difference between HIV+ and controls				
59 HIV + (all commenced PI based ART under 3 months of age, 91% VL undetectable) 43 HIV -	South Africa	7.7 (7.6 – 7.8) 8.5 (7.8 – 8.7)	PWV: after adjustment for age, gender and sBP no difference in PWV between HIV + and controls	[246]
101 HIV + (all stable on ART with VL <1000copies/ml, median (IQR) ART 3 (1 : 10) years) 86 HIV -	USA	20 (17 : 23) 19 (14 : 23)	PWV: no difference between HIV+ and HIV -. In HIV + group PWV was positively associated with systolic and diastolic BP, IMT, male sex, current alcohol use, detectable VL and current TDF use. PWV negatively correlated with CD4 count and ART duration	[247]
No controls; impact of ART				
230 HIV + (45 ART naïve, 90 EFV, 76 NVP, 19 LPV/r) No controls	Ethiopia	6 – 18 years (no mean given)	IMT: no difference between groups PWV: elevated in LPV/r-treated subjects v EFV / NVP / ART-naïve - 5.2m/sec vs. 4.7 / 4.6 / 4.6 m/sec FMD: no difference between groups	[238]
Longitudinal Study				
Pilot 31 HIV + (all on ART for median 64 months, 13 on PI) 31 HIV – Baseline 39 HIV + (34 on ART, 30 VL undetectable, 4 horizontally infected) 39 HIV – Week 48 35 HIV + 37 HIV -	USA	9 (2 -20) 9 (2 -21)	Baseline IMT significant differences between HIV+ and HIV- (ICA 0.9 v 0.78 mm, CCA 1.0 v 0.95mm), duration on ART associated with thicker IMT In HIV + significant improvements in ICA 0.73v0.65mm and CCA 0.85 v 0.9mm over 48 weeks, no significant difference between HIV+ and HIV-. Improvement in HIV – not fully explained. Duration of ART and CD4% affect change in IMT. Female sex, age LDL, ART and PI use all affect change in IM	[248, 249]
CCA, common carotid artery; EFV, efavirenz; FMD, flow mediated dilation; ICA, internal carotid artery; IMT, intimal medial thickness; LVIMP, left ventricular index of myocardial performance; LPV/r, lopinavir/ritonavir; NVP, nevirapine; PI, protease inhibitor; PWV, pulse wave velocity; TC, total cholesterol.				

2.11.1 Use of abacavir in the paediatric population

Abacavir is a key antiretroviral drug in the treatment of paediatric HIV. The PENTA 5 study proved its superiority over other first line antiretrovirals available at that time [202]. Non-inferiority, compared to d4T and ZDV, has been shown in the CHAPAS-3 study [27], suitable formulations exist and once daily dosing is an option for older children [64]. ABC remains a preferred first line NRTIs for the treatment of paediatric HIV in children under 10 years of age / 35 kg [7] as it avoids the toxicities seen with zidovudine (anaemia), stavudine (lipodystrophy) and tenofovir (renal glomerular / tubular toxicities and decreased bone mineral density). As discussed in section 2.10.8 the long term impact that ABC has on cardiovascular function in adults remains undetermined and if there is a true relationship between ABC use and increased risk of CVD then using it during the paediatric years may be the optimal time before other risk factors start to increase.

2.12 Conclusions

Paediatric HIV has been transformed since the first case studies of the 1980s to a chronic but manageable illness, where ART is available. Whilst considerable progress has been made towards the eventual goal of EMTCT by the end of the current decade, there are 2 million HIV-infected children and many more perinatally infected adolescents and adults estimated to be living with HIV.

Commencing children on ART remains the top priority, with ART optimization to minimise toxicities. The increased risk of CVD in HIV-infected adults and the potential implications of this for children living with HIV cannot be ignored. Assessment of cardiovascular health in HIV-infected young children is not easy, especially at primary or secondary care level and as yet no simple screening test has been developed for use outside of a research setting

The mechanisms underlying CVD are multifactorial and may vary by setting e.g. high versus low to middle income countries. Traditional and non-traditional risk factors contribute to increased cardiovascular risk in HIV-infected populations. The roles of inflammation and immune activation are likely to be key factors and novel ways of modulating these pathways such as the use of CCR5 antagonists,

interleukin antagonists, methotrexate and renin-angiotensin blockade are being considered.

2.13 Aims of the Thesis

The key aims of this thesis were:

1. To determine the influence of HIV infection on vascular phenotype by comparing HIV infected ART naïve children with HIV uninfected African controls

2. To determine the effects of ART on vascular phenotype by comparing children stable on ART to drug naïve about to start treatment and monitor changes in arterial stiffness over time.

3. To gain insight into the potential mechanisms operating to mediate vascular dysfunction.

2.13.1 Primary hypothesis

1. HIV infected, ART naïve children have impaired arterial structure and distensibility compared to HIV uninfected, age matched controls.

2.13.2 Secondary hypotheses

1. 96 weeks of antiretroviral therapy can improve arterial structure and distensibility in HIV infected children who were previously ART naïve.

2. Significant baseline differences in markers of inflammation, vascular injury and disordered thrombogenesis exist between HIV infected, ART naïve and HIV uninfected children.

3. 96 weeks of antiretroviral therapy can improve markers of inflammation, vascular injury and disordered thrombogenesis exist in HIV infected children who were previously ART naïve.

Chapter 3 Methods

This chapter describes the rationale behind the methods selected and the techniques used to address the specific aims of the project.

3.1 Population and study design - The CHAPAS 3 trial

HIV infected children were recruited in Zambia and Uganda as part of "The **C**hildren with **H**IV in **A**frica **P**harmacokinetics and **A**cceptability /Adherence of **S**imple Antiretroviral Regimens (CHAPAS) 3 trial". This was an open label randomised phase II/III trial (ISRCTN69078957) evaluating new formulations of solid, dispersible scored antiretroviral fixed dose combinations (FDC). CHAPAS 3 tested the hypothesis that the newly formulated FDC tablets containing abacavir (ABC) or zidovudine (ZDV) rather than stavudine (d4T) would provide superior toxicity and/or adherence/acceptability profiles in both ART naïve (group 1) and ART experienced (group 2) children, whilst maintaining adequate pharmacokinetics and similar cost-effectiveness and viral load suppression. Children were aged 13 years or younger; those recruited to group 2 had to have been on d4T containing first line ART for a minimum of two years and have an undetectable viral load (VL) at recruitment. Specific inclusion and exclusion criteria are listed in Table 3-1.

The local team verbally explained the study and recruited the children; all families were given a patient information leaflet written in English, Nyanjan (Lusaka) or Lugandan (Uganda) as appropriate. Children from each group were randomised to one of 3 treatment arms; ABC v ZDV v d4T with 3TC and either nevirapine (NVP) or efavirenz (EFV) and followed for 96 weeks. The choice of NVP or EFV was made by the clinical team; generally children aged under 3 years received NVP. Randomisation was stratified by age/ART experience, NNRTI and centre.

A total of 480 children were recruited at 4 sites (three in Uganda, one in Zambia) between the 8th November 2010 and 28th December 2011. The last child recruited reached 96 weeks follow up on the 30th October 2013. At the end of the trial children remained on ART (the ART regimen dictated by local availability/national guidelines) and continued to receive ongoing healthcare in a local clinic (where possible) or at the trial centre.

Table 3-1. Inclusion and exclusion criteria for children recruited to CHAPAS 3

Inclusion Criteria

1. Aged 1 month to 13 years
    a) ART naïve children in Uganda randomised to a d4T based regimen must be 0-4 years old in accordance with local guidelines
    b) ART experienced children randomised to continue therapy on a d4T based regimen must be ≥5 years without symptoms of lipodystrophy.
2. Weight >3kg and <25kg
3. Participants must have a confirmed documented diagnosis of HIV-1 infection
    a) <18 months: two separate peripheral blood specimens taken on different days, both must test positive with HIV-DNA PCR.
    b) ≥18 months: antibody positive serology by ELISA test (confirmed by licensed second ELISA or Western Blot) or WHO approved rapid test (performed in series) both on the same sample.
4. Parents or guardians, and children where appropriate, must be willing and able to give informed consent for randomisation to first-line ART strategy
5. **a) ART naïve** (except perinatal ART for PMPCT), meeting WHO or national (WHO modified) criteria for initiating therapy and ready to start an initial 2NRTI+NNRTI based regimen according to local guidelines.
    - WHO paediatric clinical stage IV disease: treat regardless of CD4 absolute/%
    - WHO paediatric clinical stage III disease:
        - < 12 months: treat all
        - > 12 months: treat all children irrespective of CD4 absolute/% except tuberculosis, lymphocytic interstitial pneumonia (LIP), oral hairy leukoplakia (OHP) or thrombocytopenia guided by CD4 assays
    - WHO paediatric clinical stage II or I disease: treat guided by CD4 absolute/% percent or count (as per WHO guidelines in 2010)

Criteria to start ART				
Age	**<12 months**	**12 - 35 months**	**36 - 59 months**	**≥5 years**
%CD4	All	<20	<20	<15
Absolute CD4		<750mm^3	<350mm^3	<200

b) **ART experienced**, currently taking first line d4T based regimen ≥ 2 years with:

- screening HIV RNA viral load <50 copies/ml
- no history of receiving other ART
- CD4 count and/or CD4 percent stable over the previous 6 months.

Exclusion Criteria

1. Cannot, or unlikely to attend regularly (e.g. lives too far from study centre)
2. Likelihood of poor adherence
3. Presence of acute infection (e.g. malaria, helminthiasis, acute hepatitis, acute pneumonia, septicaemia, meningitis). Children may be admitted after recovery of an acute infection. Children with chronic lung disease, including recurrent respiratory infections, are eligible. Children with tuberculosis (TB) not be enrolled during the intensive phase of TB treatment.
4. In receipt of medication contraindicated by ART
    - Children <3 years on TB treatment should not be enrolled (as they will have to receive nevirapine).
    - on chemotherapy for malignancy
5. Laboratory abnormalities which are a contra-indication for the child to start ART/change to any of the 3 possible regimens (haemoglobin <8.5g/dL; neutrophils <0.50x10^9/L; AST or ALT >5 x the upper limit of normal (ULN); grade 3 renal dysfunction - creatinine >1.9 x ULN).
6. Being pregnant or breast-feeding an infant
7. Perinatal exposure to NVP (either through PMTCT or breastfeeding) for children aged 3 - 6 months only

3.1.1 Ethical approval and consent

The CHAPAS 3 trial including cardiovascular sub-study has full ethical approval from University College London (1665/002), Baylor College of Medicine, Uganda (H-27028), Joint Clinical Research Centre, Uganda, National Drug Authority, Uganda (293/ESR/NDA/DID-12/2010), Uganda National Council for Science and Technology (HS 774), University of Cape Town (143/2010), Pharmaceutical Regulatory Authority, Zambia (DMS/105/1/112) and The University of Zambia (012-01-09).

Parents / guardians of all children included in the study provided written informed consent for study entry. Older children deemed competent provided informed consent / assent as appropriate. Example consent forms are given in appendix 1. Families / guardians received reimbursement for their travel expenses and children received a meal at clinic visits which required them to be fasted but no financial incentives were given for participation in the study.

3.1.2 Funding

The CHAPAS 3 trial was funded from a European & Developing Countries Clinical Trials Partnership (EDCTP) award. The cardiovascular sub-study was fully funded by a Wellcome Trust Research Training Fellowship competitively awarded to myself.

3.1.3 Clinical data management

All CRFs were double entered locally by the data entry team to a central database held at the MRC Clinical Trial Unit. All patients and controls were anonymized and the database was password protected.

3.2 Selection of methods to assess the structure and arterial stiffness of the vascular system

Several methods of assessing early pre-clinical changes in the cardiovascular system have been described including coronary artery calcification, flow mediated dilation (FMD), intimal medial thickness (IMT) and pulse wave velocity (PWV). Given the age, size and number of the children that were to be assessed annually, investigations had to be quick, harmless (not involving any radiation) and painless. The techniques had to be simple allowing a number of local doctors and nurses at

each site to be trained to a high standard with acceptably low inter and intra operator variability. Any required equipment had to be affordable, transportable and sufficiently robust to cope with hot, humid and dusty environments.

Coronary artery calcium scores and coronary computed tomographic angiography been described in adult populations and provide tools to assign cardiac risk in adults. However the equipment required (a computerised tomography (CT) scanner), the radiation exposure and lack of evidence of any detectable changes in young children meant these techniques were not appropriate for this study [250]. FMD, whilst useful in assessing endothelial dysfunction [251, 252], requires patients to lie very still for a minimum of 10 minutes and is very sensitive to environmental and lifestyle factors such as ambient temperature [253], recent food consumption [254], mental stress [255] and intercurrent illnesses [256]. For these reasons using FMD was deemed unfeasible for this very young population.

IMT is a measurement of the thickness of tunica intima and tunica media, the innermost two layers of the artery wall (Figure 3-1), IMT provides a method of detecting pre-clinical thickening which is described in the early stages of the atherosclerotic process [257]. It is measured by non-invasive high resolution B mode ultrasonography. In B-mode (brightness mode) ultrasound, two-dimensional images are produced from a linear array of transducers simultaneously scanning across a plane. In adults, IMT has been extensively measured in different populations and disease states; it can be related to cardiovascular risk and used as a surrogate endpoint for evaluating the regression and/or progression of atherosclerotic cardiovascular disease [258-262]. A relationship between measured IMT and histological findings has been described [263] and improvements in IMT following therapeutic interventions with anti-hypertensive medication, lipid lowering agents and strict diabetic control reported [264-266]. IMT is influenced by age, ethnicity, diabetes mellitus [267, 268], dyslipidaemia [269, 270], hypertension [271, 272] cholesterol, BMI and smoking [273].

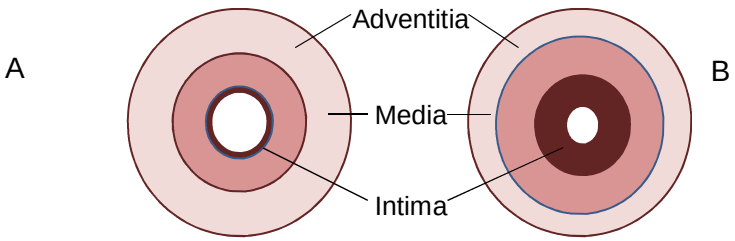


Figure 3-1. Cross section through an artery.

A) a normal artery showing the 3 layers of the arterial wall and B) early changes in the atherosclerosis process are thickening of the intimal and medial layers with narrowing of the lumen.

In paediatric/adolescent studies an increased aortic IMT has been demonstrated in children at high risk of developing atherosclerosis [274] and known cardiovascular risk factors are associated with increased carotid IMT [275-279]. As described in section 2.11 and detailed in table 2.8 a number of studies looking at IMT in older HIV infected children have been carried out with conflicting results.

An increase in arterial stiffness has been demonstrated early in the process of atherosclerosis. PWV is a well-established non-invasive reproducible technique for measuring arterial stiffness [280-282]. PWV can be affected by ambient temperature, movement and recent food consumption, however is relatively simple to perform and if performed carefully, confounding factors can be minimised. An increase in PWV is strongly correlated with cardiovascular risk factors and events [283] and all-cause mortality [284-286]. HIV infection has been demonstrated to have a detrimental impact on PWV in HIV-infected adults [86, 287] and as detailed in section 2.11 and table 2.8 in children [238, 241].

IMT and PWV were therefore felt to be the most practical and robust techniques to use for this study. Whilst no published data existed in children less than 5 years of age at the time the study was being designed, it was considered feasible to attempt

the these techniques in children of all ages. The sample size was sufficient to absorb loss of data from a predicted significant proportion of the younger children whom may be un-cooperative with the scanning techniques.

3.3 Technique to measure Intimal Medial Thickness

Whilst theoretically any artery can be used to measure IMT, the carotid artery is most frequently used. Peripheral arteries such as the femoral, brachial and radial arteries are more muscular and changes in IMT are more difficult to interpret [288]. The common carotid artery (CCA), 1 -2 cm proximal to the carotid bulb runs close and parallel to the skin surface, so it is easier to obtain clear images and accurate measurements. The internal carotid artery (ICA) and carotid bulb are not studied as frequently as obtaining measurements is more technically demanding especially in young children with shorter necks [289, 290]. Atherosclerotic lesions appear later in the CCA compared to within the ICA or bifurcation although thickening at all sites is equally associated with risk of subsequent cardiovascular events [261, 266]. IMT measurements can be taken from either the near or the far wall of the artery; far wall measurements are generally preferred as obtaining reliable and reproducible images of the near wall are dependent upon gain settings and are more difficult to standardise. Reproducibility of IMT measurement is improved if the mean, rather than the maximum IMT is measured and if automated software for analysis is used [291].

The protocol followed was the standard protocol used by the Vascular Physiology Department, University College London [241]. A robust and portable ultrasound machine, the Sonosite MicroMaxx® (FUJIFILM SonoSite, Washington) was used at both sites - Figure 3-2.

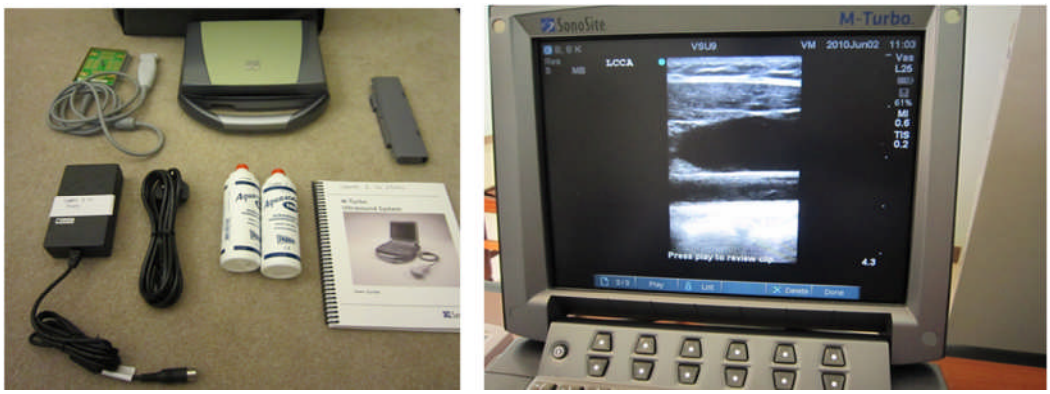


Figure 3-2. The Sonosite Micromaxx portable ultrasound machine.

Following an explanation of the investigations, patients lay semi prone / supine either on an examination couch or in their carers' arms. Ambient temperature was recorded and children were distracted using hand puppets. Using a 13-6 MHz Transducer the carotid bulb was identified and the segment of the common carotid artery 1cm proximal to the bulb located. The transducer was then placed parallel to the carotid artery with the lumen maximised in the longitudinal plane. The probe was gently repositioned until an image was obtained that was sufficiently centred and clear – as demonstrated in Figure 3-3. The image had to allow quantitative measurements of:-

a) The vessel interfaces; the near and far wall adventitia-periadventitia, media-adventitia, lumuna-intima of both common carotid arteries, and carotid bulbs.

b) The wall thickness; including mean and maximum intimal-medial thickness of near and far walls of both common carotid arteries.

c) The vessel width; including mean and maximum width of the common carotid arteries.

Once an optimal image was obtained, a minimum of 3 separate 10 second recordings were saved in **D**igital **I**mages and **Co**mmunication in **M**edicine (DICOM) format. These were downloaded for transfer to the UK for subsequent offline analysis using an automated computerized edge tracking package (Carotid Analyzer

for Research (Medical Imaging Applications, Iowa, USA)). Carotid IMT was calculated as the distance between the first bright line (luminal-intimal interface) and the leading edge of the second bright line (medial-adventitial interface) as demonstrated in Figure 3-4. Since the IMT of the carotid artery changes throughout the cardiac cycle (maximal during diastole), the three measurements of the right and left CCA at the end of diastole from three different frames were averaged [241]. For a cIMT scan to be accepted, all readings (end diastolic diameter, systolic diameter and end-diastolic IMT) from both sides had to be with 0.1mm of each other. If a medical practitioner was only able to obtain an analysable scan on one side, the mean of 3 readings from that side was calculated.

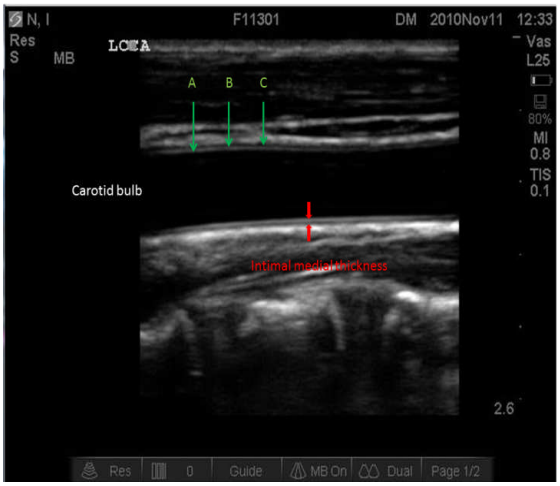


Figure 3-3. Ultrasound image of the left common carotid.

A, intima; B, media; C, adventitia

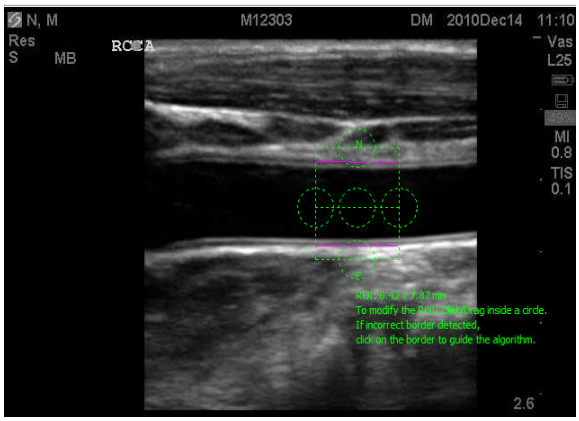


Figure 3-4. Calculation of cIMT using Carotid Analyzer for Research automated software.

3.4 Technique to measure Pulse Wave Velocity

PWV was measured after the IMT. The majority of babies and children had been fasted for at least an hour prior to the PWV being performed. Where this was not possible the time of last milk / meal was documented. Recordings were performing with the children lying in the semi-prone position (at approximately 30°) to prevent venous contamination of the arterial signal. The PWV was measured using a Vicorder (Skidmore Medical, Bristol, UK) - Figure 3-5.

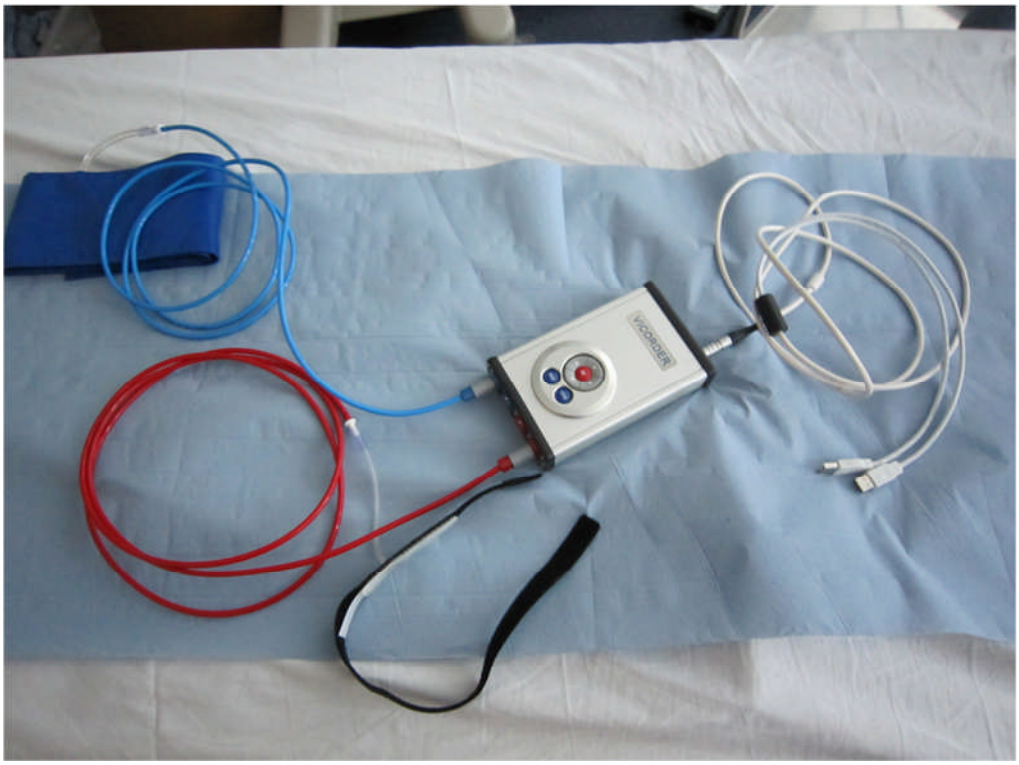


Figure 3-5. Picture of the Vicorder used to measure the Pulse Wave Velocity

The carotid artery was palpated and a 30mm neck cuff placed where the carotid artery pulse could be palpated. An appropriately sized femoral cuff was placed proximally on the thigh, as high as possible. Three distances were recorded using a paper tape measure to the nearest mm:

a) Distance between the base of the carotid cuff and suprasternal notch

b) Distance from the top of the suprasternal notch to the middle of the thigh cuff

c) The direct measurement between the base of the carotid cuff to the middle of the thigh cuff.

Once the cuffs were adequately positioned they were simultaneously inflated to 60mmHg. Ten seconds of waveform data were recorded once a reproducible signal was obtained, Figure 3-6, and the integral software calculated the difference in speed from the upstrokes of the waveforms. A PWV was calculated on this difference, and the arterial path length between the two sites [242]. PWV was calculated as the distance: transit time ratio and expressed as meters per second (m/sec). For each subject at least 3 PWV readings within 0.5m/second were recorded and the mean calculated.

To date no consensus has been reached as to how best to standardize measurement of the arterial path [292]. For this study direct measurements from the suprasternal notch to the middle of the femoral cuff were used. This excludes the proximal segment of the aorta and includes a variable length of iliac and femoral arteries [284, 286]. Two other methods of measurement have been described. Firstly, direct measurements from the base of the carotid cuff to the top of the femoral cuff and then subtracting the distance from the base of the carotid cuff to the suprasternal notch [293]. Alternatively measuring the distance between the carotid and femoral sites directly, and applying a correction factor of 0.8 has been suggested which appears to fit better with actual path length distance, measured from MRI [294]. The different methods used may give different values. PWV results obtained using a direct measurement between carotid and femoral cuffs will be higher than those using the other methods used. This may limit comparison with other cohorts or its utility for clinical use to stratify vascular risk. For this study all

measurements were recorded so that adjustments could be made retrospectively if needed.

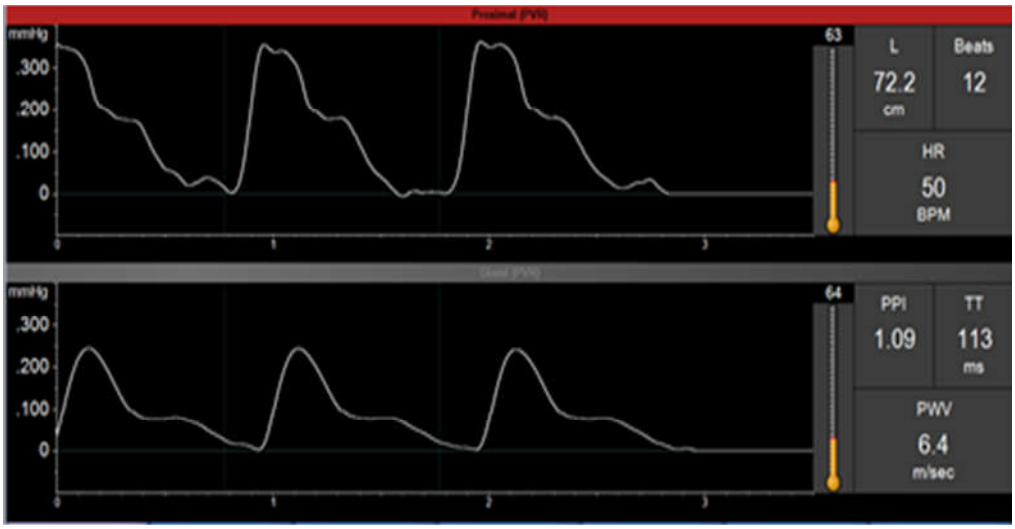


Figure 3-6. Example of a PWV trace obtained.

The carotid waveform is displayed in the top half of the screen, the femoral waveform is displayed in the bottom half of the screen. The waveforms are consistent beat to beat with the amplitude optimised

3.5 Blood pressure measurement

Accurate measurement of blood pressure (BP) can be difficult in childhood. Options include a manual sphygmomanometer or automated oscillometric devices such as the Omron M2 (Omron Healthcare, Milton Keynes, UK), which have been validated in children. The automated machines have the advantage that they are smaller, portable and are without observer bias. The Omron M2 was chosen after ensuring paediatric validation with the Association for the Advancement of Medical Instrumentation (AAMI), The British Hypertension Society working party on BP measurement and the European Society for Hypertension [295].

3.6 Biomarkers

3.6.1 Rationale behind biomarkers selected.

As will be discussed further in Chapter 6.1 and summarised in table 6.1 a large body of literature exists describing the changes in biomarkers with HIV infection and the use of these markers in HIV disease progression and cardiovascular risk assessment. I set up a panel of 19 biomarkers to be studied in several trials within Professor Klein's HIV laboratory. Together they form a comprehensive panel covering markers of inflammation (IL-1Ra, CRP, TNFa, IL-10, IL-6, IL-8, serum amyloid A (SAA) and ICAM-3), cardiovascular injury (MCP-1, Angiopoeitin-1 and 2, E-selectin, P-selectin, sICAM-1, VCAM, VEGF) and disordered thrombogenesis (d-dimer, thrombomodulin (TM) and tissue factor (TF)).

3.6.2 Rationale behind methods selected.

The blood volume that can be collected in children is limited, which influenced the techniques selected for this study. The assay selection was also influenced by the robustness to freezing at -70^{o}C for variable periods of time. Multiple assays had to be able to be run on one day to avoid repeat freeze-thaw cycles.

3.6.3 Laboratory methodology

Blood was collected in **E**thylene**d**iamine**t**etra**a**cetic acid (EDTA) and spun within 4 hours of collection at 1500g (≥2500 rpm) for 15 minutes to separate cells from

plasma. The supernatant was removed and placed in aliquots with a minimum of 500µL of plasma in each cryovial. Samples were immediately frozen at -70^{o}C.

All samples were transported on dry ice to the Institute of Child Health, UCL and the assays ran in batches. All standards were run in duplicate. Initial work within our lab showed that the co-efficient of variance (CV) between duplicate samples was ≤10%. Given the small volumes of blood available and the cost of the reagents and consumables, 95% samples were run singularly, with 5% duplicated to ensure consistent intra and inter assay precision. The lower limit of detection, determined by the mean +2 standard deviations of the output signal of 10 blank samples, was calculated for each biomarker.

3.6.4 Meso scale discovery (MSD) technique

A total of 17 biomarkers were analysed using Meso Scale Discovery (MSD) assays (MesoScale Discovery, Gaithersburg, MD, USA). MSD assays use an electrochemiluminescence detection method similar to a sandwich ELISA technique. Electrochemiluminescent (SULFO-TAG) labels are conjugated to detection antibodies. The microplates have high binding carbon electrodes in the base. Within the analyser, electricity applied to the plate electrodes causes light emission by the SULFO-TAG labels. Light intensity is then measured to quantify analytes in the sample. Multiple excitation cycles of each label amplify the signal to enhance light levels and improve sensitivity allowing ultrasensitive assays to be run [296].

Five MSD plates were used, 3 of which were custom built for this study, Table 3-2**Error! Reference source not found.** summarises the details of the plates used. Assays were conducted according to standard manufacturer's protocols. Samples were read using the QuickPlex SQ analyser (Meso Scale Discovery®, Gaithersburg, MD, USA).

Table 3-2. Summary of Biomarkers measured.

Method used for detection and lower limit of detection per assay

Method	Plate	Biomarker	Units	Lower limit of detection
MSD	Vascular injury-1	TM	ng/ml	0.064
		ICAM-3	ng/ml	0.064
		E-SEL	ng/ml	0.064
		P-SEL	ng/ml	0.064
	Vascular injury-2	SAA	ng/ml	10.9
		CRP	ng/ml	1.33
		VCAM-1	ng/ml	6
		ICAM-1	ng/ml	1.03
	Custom cytokine	IL-6	pg/ml	0.192
		IL-8	pg/ml	0.132
		IL-10	pg/ml	0.0806
		MCP-1	pg/ml	0.116
		TNFa	pg/ml	0.0798
		VEGF	pg/ml	0.229
	IL-1Ra	IL-1Ra	pg/ml	2.44
	Ang 1+2	Ang-1	pg/ml	24.4
		Ang-2	pg/ml	2.44
ELISA	TF	TF	pg/ml	0.69
	D-dimer	D-dimer	ng/ml	0

IL-1Ra, interleukin-1 receptor antagonist; CRP, C reactive protein; TNFa, tumour necrosis factor A; IL-10, interleukin-10; IL-6, interleukin-6; IL-8, interleukin-8; ICAM-3, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; Angio 1, angiopoietin-1; Angio 2, angiopoietin-2, Ang 2: Ang 1, ratio of angiopoietin 2:1; sICAM, soluble intracellular adhesion molecule; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; TF, tissue factor; TM, thrombomodulin.

3.7 Tissue factor

Tissue Factor (TF) levels were measured using a commercial quantitative sandwich Enzyme-Linked Immunosorbent Assay (ELISA) kit (Quantikine® ELISA Human Coagulation Factor III/Tissue Factor, R&D Systems, Minneapolis). 100 µL of assay diluent (R&D) was added to 96-well microplates pre-coated with a monoclonal antibody against human tissue factor. Subject plasma was diluted two-fold in Calibrator diluent RD5-20 (R&D). 100uL standards (R&D) and diluted subject plasma were added and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 600 rpm. The plate was manually washed 4 times using Wash Buffer (R&D), and excess fluid removed by tapping the plate on a paper towel before 200 µL of Tissue factor enzyme linked polyclonal antibody conjugate was added. After further 2 hours incubation the wash cycle was repeated and 200 µL of substrate solution (stabilized hydrogen peroxide / chromogen (tetramethylbenzidine) R&D) added. The plate was then protected from light during the final 30 minutes incubation after which 50µL of stop solution (2N Sulfuric Acid, R&D) was added and optical density was measured within 30 min using a microplate reader (Thermo Multiskan EX) set to 450nm. A four parameter logistic curve fit was calculated using Ascent software 2.6 (Thermo Labsystems Oy) and the concentration read from the standard curve and multiplied by the dilution factor of 2.

3.8 D-dimer

The commercial TECHNOZYM® D-dimer ELISA assay (Technoclone, Austria) was chosen following a comparison of 2 ELISA methods with 2 automated methods used in the NHS coagulation laboratory at Great Ormond Street Hospital. The TECHNOZYM assay provided consistent results and required smaller volumes of blood than the automated methods.

Undiluted samples were used after pilot runs showed most patients had levels below the limit of detection when a 2 fold dilution was used. 100ul of calibrator and sample were added to wells pre-coated with anti D-dimer monoclonal antibody then incubated at 37^{o}C for 60 minute. Following manual plate washing 3 times using wash buffer (Technoclone – PBS; pH 7.3) 100ul conjugate (monoclonal Anti D-dimer-POX) working solution was added before further 60 minute incubation at

37^{o}C. Following a second plate wash 100ul substrate solution (Chromogen tetramethylbenzidine) was added. After a 10 minute incubation at room temperature 100ul stop solution (sulphuric acid) was added and the plate read immediately using a microplate reader (Thermo Multiskan EX) set to 450nm. A linear regression curve fit was calculated using Ascent software 2.6 (Thermo Labsystems, Helsinki, Finland) and the concentration read from the standard curve.

3.9 Immunophenotyping

3.9.1 Immunophenotyping background

The concept of the role of immune activation in the pathogenesis of AIDS is not new [297-299]. Strong indirect evidence comes from studies of Simian Immunodeficiency Virus (SIV) infections in Rhesus macaque and Sooty mangabey monkeys. SIV infected Sooty mangabey monkeys whom, despite high levels of viral replication, have low levels of immune activation and do not progress to AIDS. Conversely SIV infected Rhesus macaque monkeys have increased levels of immune activation and progress to AIDS [93, 300-303]. Assessment of immune activation and proliferation can be made through measuring HLA-DR, CD38, CD45RA/RO and Ki-67, on T cell subsets [304-306]. Human studies have described that excessive or uncontrolled immune activation, evidenced by high levels of activated CD8 T-cells expressing CD38 and HLA-DR, correlate with CD4 death [307] and disease progression more closely than viral load [308-311].

To characterise the extent of immune dysfunction in individual patients 2 panels were used looking specifically at activation and proliferation markers - Table 3-3.

Table 3-3. T cell markers studied

Marker	Function	Effect of HIV	Ref.
Activation panel			
HLA-DR	A MHC class II cell surface receptor that is up-regulated on activated T cells	Related to viral load A sensitive marker of disease progression	[312]
CD38	A transmembrane glycoprotein expressed by T, B and dendritic cells, associated with intracellular signalling A T-cell activation marker but is very person and disease specific, changes can be seen with intercurrent infections and with disease progression.	CD8+CD38+ strongly related to disease progression in ART naïve patients. On ART levels abnormally high despite VL suppression, which are of unknown significance but may represent a CD38+ compartment enriched in naïve recent thymic emigrants (CD45RA+) CD8+CD38-HLA-DR+ identified in elite controllers and have a protective function	[94, 309, 310, 313-317]
Proliferation panel			
CD45	Leukocyte common antigen. 2 isoforms RA/RO. CD45RA+ are naïve cells, CD45RO+ are memory cells having been exposed to antigen. At birth >90% are naïve cells the proportion of which progressively decreases until 18 years of age by which 50% naïve : 40% memory cells.	With HIV infection memory cells lost more than naïve in adults. After commencing ART in adults initially redistribution of memory cells then an increase in naïve cells Post ART initiation in children increase mainly in naïve component With treatment interruption CD4 expression of RA and RO remains constant in children	[47, 48, 318, 319]
CD31	Also known as platelet endothelial cell adhesion molecule-1 CD31+ recent thymic emigrants, fall with age. CD31- have proliferated in the circulation	With ART CD31+CD45RA+ see similar levels to controls, CD31-CD45RA+ remain lower than controls.	[320]
Ki67	A nuclear cell cycle antigen Expressed preferentially during all active phases of the cell cycle but absent in resting cells (G0), making it a useful marker of proliferation	In ART naive individuals up to 50% of the circulating cells may be proliferating then dying ART reduces proportion of proliferating cells	[321]

3.9.2 Whole blood flow cytometry staining protocol

To assess activation an activation panel was set up to subdivide CD4+ / CD8+ cells into 4 populations based upon expression or lack of HLA-DR and CD38 and to quantitatively detect the percentage of cells within each of these populations that expressed HLA-DR. Within 4 hours of collection whole blood was incubated for 30 minutes at room temperature with 4 antibodies; anti-HLA-DR, anti-CD3, anti-CD8 and anti-CD38 - Table 3-4. Appropriate isotypic controls (mouse IgG1-PE and IgG2b-APC) were used to evaluate non-specific staining. Cells were then washed with Automacs Buffer (Milteny Biotec Inc. Auburn, CA, USA) to remove unbound antibody and lysed red blood cells and re-suspended in Phosphate buffered saline ((PBS), Invitrogen Ltd, UK) supplemented with 1% paraformaldehyde.

Table 3-4. Antibodies used in flow cytometric analysis.

Human target molecule	Mouse isotype	Conjugate	Source
Activation panel			
HLA-DR	IgG2b	APC	BD Biosciences UK
CD3	IgG	PerCP	BD Biosciences UK
CD8	IgG1	FITC	BD Biosciences UK
CD38	IgG1	PE	BD Biosciences UK
Proliferation panel			
CD4	IgG1	PerCP	BD Biosciences UK
CD45RA	IgG2b	APC	BD Biosciences UK
CD31	IgG1	PE	BD Biosciences UK
Ki67	IgG	FITC	BD Biosciences UK

The source, mouse isotype and flurochrome conjugation used are detailed. FITC - fluorescein isothiocyanate, PerCP - peridinin chlorophyll protein, PE - phyoerythrin, APC – allophycocyanin.

To assess proliferation, a flow cytometric assay was used to subdivide CD4+ T cells into four populations based on expression of CD45RA and CD31 and to quantitatively detect the percentage of cells within each of these populations that expressed Ki67. Within 4 hours of collection whole blood was incubated with 3 antibodies: antiCD4, anti-CD45RA and anti-CD31 with appropriate isotypic controls (mouse IgG1-PE and IgG2b-APC) - Table 3-4. The red blood cells were lysed and washed with Automacs Buffer. The remaining cells were fixed then permeabilised with Perm Buffer containing Saponin (Sigma-Aldrich, UK). Saponin was chosen as it does not destroy the cell or affect membrane expression of surface antigens. Cells

were then incubated with anti-Ki67 antibody for 30 minutes. The excess antibody was removed by washing with Perm buffer followed by FACS buffer the cells were re-suspended and fixed in Phosphate buffered saline ((PBS), Invitrogen Ltd, UK) supplemented with 1% paraformaldehyde.

All samples were analyzed by four-color flow cytometry using a FACS Calibur (Becton-Dickinson) with a 488 nm argon-ion laser and a 635 nm red diode laser. 50,000 events were collected in the lymphocyte gate using morphological parameters (Forward and Side scatter). Data were processed using CellQuest Pro Software (Becton-Dickinson).

Data was stored and transferred to the UK. Consistency was ensured by a single observer re-analysing all the data. Flow Jo software version 7.6.4 (Treestar Inc, California, USA) was used and the methods used for analysis were in agreement with techniques used in the clinical laboratories at Great Ormond Street Hospital.

3.9.3 Identification of activated lymphocytes

Forward and side scatter were used to identify the lymphocytes, monocytes and neutrophils to which tight gates were applied Figure 3-7 i). A gate was applied to the monocyte population, and the proportion expressing DR used to set the gates for subsequent analysis. If a clear boundary could be identified on a dot plot this was used; for indistinct plots, a contour plot helped place the gate at the point where the contours start to space out.

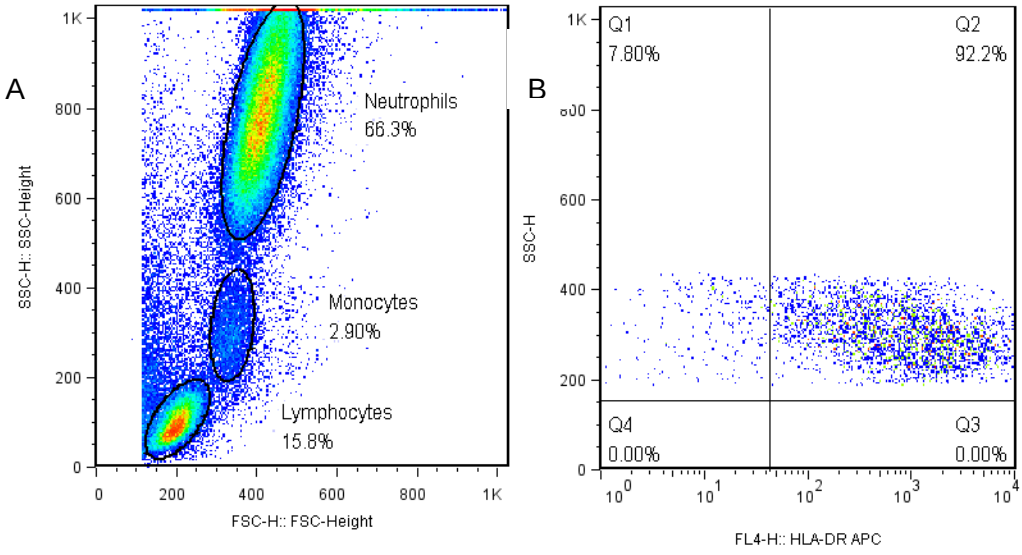


Figure 3-7. Identification of activated lymphocytes.

A. Plot of forward against side scatter height, tight gates applied to each cell population (lymphocytes, monocytes and neutrophils). Monocyte population were then selected B. plot of monocyte population; gated to identify the HLA-DR+ population, this gating was used for subsequent analysis.

The percentage of monocytes positive for HLA-DR (quadrant 2 (Q2) was recorded along with the mean fluorescence intensity (MFI) of HLA-DR - Figure 3-9. The lymphocyte populations were then selected and the CD3+ cells divided into populations of CD4+ and CD8+ cells - Figure 3-8. The CD4 and CD8 populations were divided into 4 sub populations based on the expression of HLA-DR and CD38 - Figure 3-10 using the same gate set after gating on DR expression by monocytes.

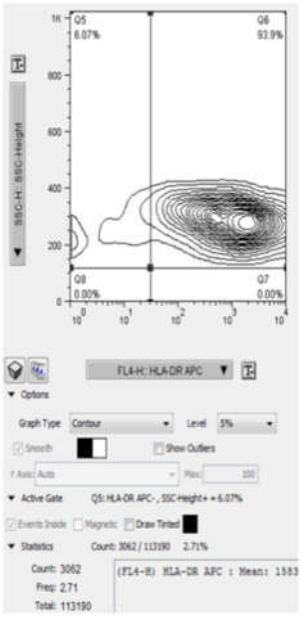


Figure 3-9. Using the monocyte population to set the DR gate

The percentage of DR+ monocytes (Q2) and MFI of the HLA-DR were then able to be calculated.

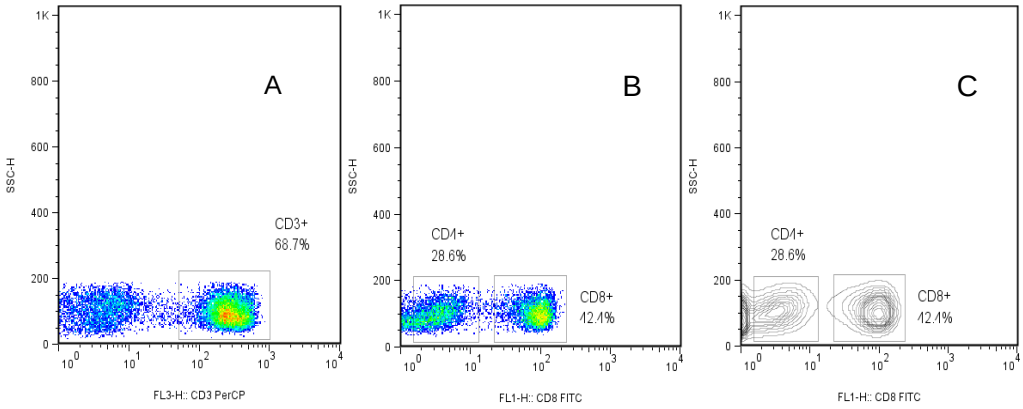


Figure 3-8. Using the lymphocyte population to separate into CD4+ and CD8+ lymphocytes.

A) Gating on the lymphocytes the CD3+ population were selected

B) and C) CD3+ cells were divided into CD8- (CD4+) and CD8+

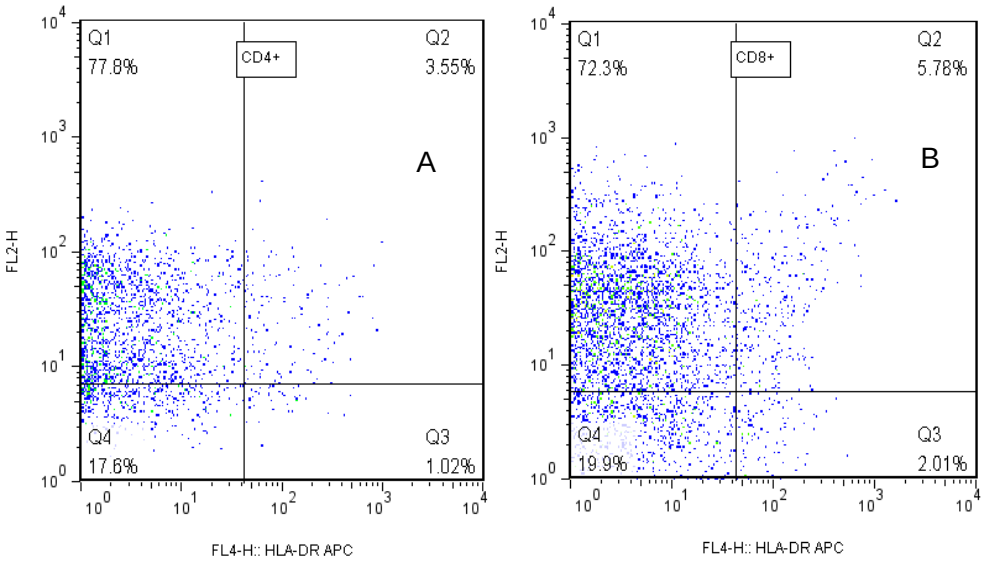


Figure 3-10. Using the expression of HLA-DR and CD38 to sub divide into 4 populations

Dividing A) CD4 and B) CD8 cells into 4 sub populations based upon the expression of HLA-DR and CD38

3.9.4 Analysis of proliferation data

Forward and side scatter were potted to identify the lymphocyte population. The lymphocytes were gated Figure 3-11 *A)* and the CD4+ population Figure 3-11 *B)* then subdivided into 4 populations based upon the expression of CD31 and CD45RA, Figure 3-12.

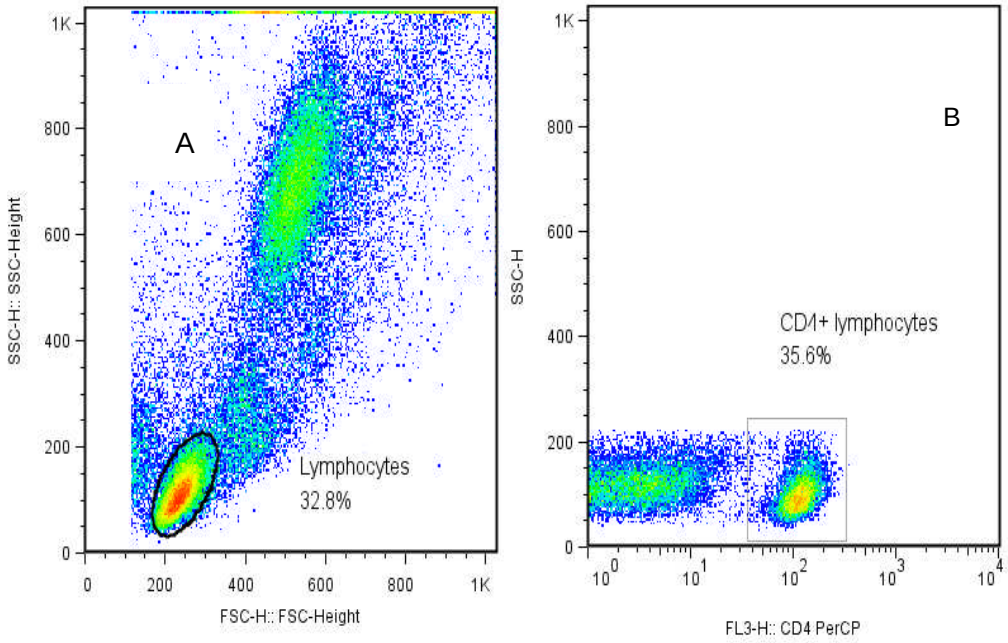


Figure 3-11. Identification of the CD4+ lymphocyte subpopulation.

A) Using forward and side scatter to identify the population of lymphocytes B) CD4+ lymphocytes selected.

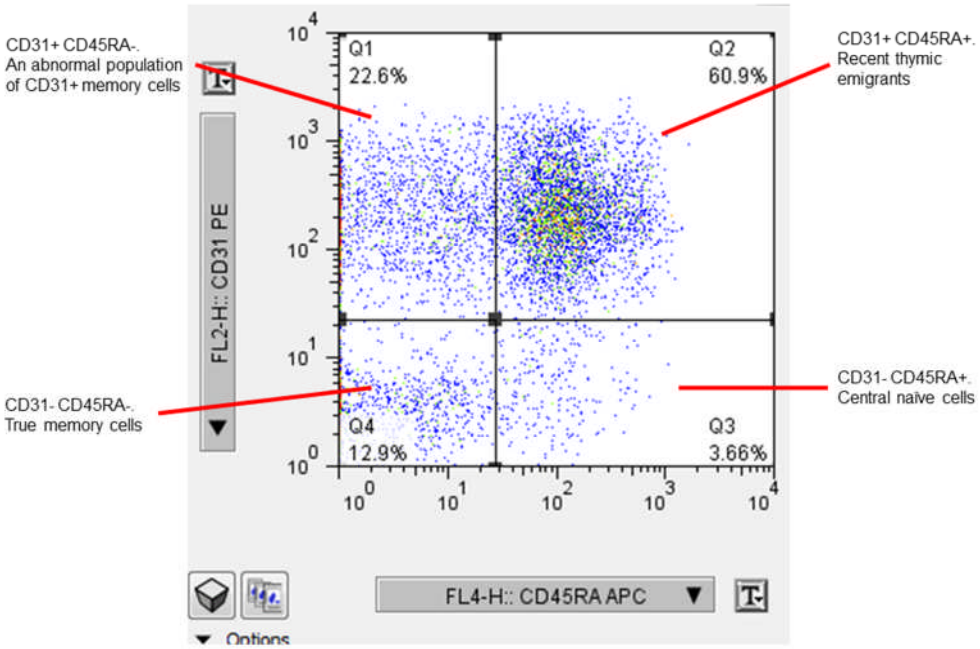


Figure 3-12. Diving CD4+ lymphocytes into 4 subpopulations based upon the expression of CD31 and CD45 RA.

The proportion of proliferating cells, as evidenced by expression of Ki67, was then quantified in the total CD4+ lymphocyte population as well as in each sub-population. The gates were applied using the dot plot and contour plots adding the gate where the contour lines become less dense, Figure 3-13.

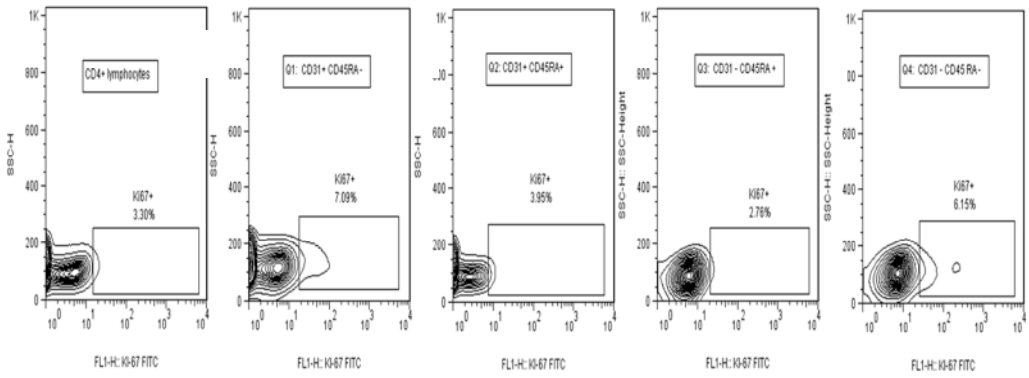


Figure 3-13. Quantifying the proportion of Ki67 + cells

A) CD4+ lymphocytes, B) CD31+ CD45RA- lymphocytes, C) CD31+ CD45RA+ lymphocytes, D) CD31- CD45RA+ lymphocytes, E) CD31- CD45RA- lymphocytes.

3.10 Missing data

For the cardiovascular scans (IMT/ PWV) incomplete baseline data was mainly due to the young age of children. The age range of HIV-infected / controls with cardiovascular scan data remained balanced and further analyses took account of missing data. Less than 5% of patients had missing biomarker results at any time point whilst technical difficulties with the IPT data meant that <24% samples at each time point were missing but subsequent analyses were adjusted for this.

3.11 Statistics

Comparison of categorical variables was performed using Chi squared test or Fishers exact test (depending upon sample size). Normally distributed data is expressed as mean +/- SD, where normality cannot be assumed, the median with the range or interquartile range (as appropriate) will be stated. Parametric tests were used whenever possible (i.e. when the data is normally distributed, or can easily be transformed to normality). Normality of the data was assessed by plotting histograms and applying the Kolmogorov-Smirnov test of normality. When the data could not be transformed, non-parametric tests were performed. The differences in the distribution of non parameteric parameters were tested using the Mann-Whitney test (2 groups) or Kruskal-Wallis (3 or more groups). Associations between 2 continuous variables were tested for statistical significance using the Spearman's rank correlation test. Comparisons of related variables in the same subject were performed using the Wilcoxon signed rank test. All tests for statistical significance were two-tailed and p valves <0.05 were considered significant. Repeated measurements collected from the study population linear mixed effects models were used to assess the association of IMT and PWV with clinical and sociodemographic characteristics of the HIV infected study population. Multiple regression analysis, backward elimination selection was used to determine variables independently associated with cardiovascular parameters. P values and coefficients they were rounded to 2 decimal places / 1 significant figure. Stata 13 analysis package (Statacorp. College Station, TX) was used for data analysis.

Chapter 4 Establishing the cardiovascular sub study

4.1 CHAPAS 3: The Cardiovascular Sub study

The CHAPAS 3 cardiovascular sub study was established at 2 of the 4 CHAPAS 3 sites; University Teaching Hospital (UTH), Lusaka, Zambia and the Joint Clinical Research Centre (JCRC), Kampala, Uganda (see Chapter 3 for details of the CHAPAS 3 study). The funding available only permitted recruitment to the cardiovascular sub-study at 2 of the 4 sites so the larger sites that had capacity to employ additional staff were selected. All 282 children recruited at these 2 sites were consented and enrolled into the cardiovascular study at the same time as enrolment to the main CHAPAS 3 study; no children were excluded. In addition 284 age-matched HIV uninfected control children were recruited from community well child clinics, surgical outpatient clinics and healthy siblings of CHAPAS 3 patients. Eligible controls (for inclusion / exclusion criteria see Table 4-1) were approached by the local study team, the study explained verbally and a patient information leaflet was provided in English, Nyanjan (Lusaka) or Lugandan (Kampala) as appropriate. To confirm HIV status all children had an HIV 4th generation antibody test performed. If this was positive and the child was aged less than 18 months of age a HIV DNA PCR was performed. If either the 4th generation antibody test (>18 months) or the HIV DNA PCR (<18 months) was positive a repeat confirmatory test was performed on a second sample. If a child was found to be HIV-infected the child was referred for routine HIV care. The control children were assessed once and not followed up.

CHAPAS 3 recruits were seen at screening, baseline (week 0), 2 weeks, 6 weeks then at a minimum of 12 weekly intervals until the end of the trial (defined as the last patient recruited reaching week 96 – 30th October 2013). The cardiovascular assessment is detailed in Table 4-2. Medical and family histories were obtained, specifically documenting personal and family history of cardiovascular disease, birth weight/gestation and parental tribe. Anthropometric measurements, skin fold thickness and body circumferences were measured every 24 weeks. HIV disease activity was assessed by general health, clinical examination and monitoring of CD4 count as part of the CHAPAS 3 protocol. A full blood count, renal and liver function tests were

performed 24 weekly. An additional 5ml of blood was taken at 0, 48 and 96 weeks for extended cardiovascular assessment. Blood was processed immediately for 2 immunophenotyping (IPT) panels looking at markers of immune activation and proliferation (see section 3.9). The remainder of the blood was collected into EDTA and citrate tubes, spun within 4 hours of collection and frozen at -70^{o}C for later batch analysis of biomarkers of systemic inflammation, disordered thrombogenesis and cardiovascular damage. Vascular assessment using non-invasive techniques to measure cIMT and PWV were performed at 0, 48 and 96 weeks. Examples of the consent and clinical report forms (CRFs) used are in appendix 1.

Inclusion Criteria

1. Aged 1 month to 13 years
2. Weight >3kg and <25kg
3. Parent / guardian and children (where appropriate according to age and knowledge of HIV), must be willing and able to give informed consent for HIV testing and cardiovascular assessment
4. Confirmed HIV-1 uninfected
    a. All children aged ≥18 months: antibody negative serology by ELISA test or WHO approved rapid test
    b. For children aged <18 months: either antibody negative serology by ELISA test / WHO approved rapid test or a negative HIV-DNA PCR.

Exclusion Criteria

1. Presence of acute infection (e.g. malaria, helminthiasis, acute hepatitis, acute pneumonia, septicaemia, meningitis).
2. Children with tuberculosis (TB) during the intensive phase of anti-tuberculosis therapy
3. Being pregnant or breast-feeding an infant

Table 4-1. Inclusion and exclusion criteria for HIV uninfected children recruited to the control arm of the cardiovascular sub-study.

Table 4-2. Overview of the CHAPAS-3 cardiovascular sub-study.

Parameter			Frequency	Rationale for inclusion in data collection
History	Gestation and birth weight		Baseline	Low birth weight a RF for CVD
	Tribal descent			Genetic RF for CVD
	Malnutrition		Baseline and every visit	Rapid re-feeding a potential RF for later development of
	Previous and current WHO staging			Assessment of severity of HIV disease
	Family history of cardiovascular disease		Baseline and end of trial	Genetic RF for CVD
Examination	Weight and height		Every visit	Assessment of general health, obesity a risk factor for CVD
	Full examination		6 monthly (more if indicated)	
	Blood pressure		Week 0, 48, 96	Hypertension a RF for CVD
	Assessment of lipodystrophy			Lipodystrophy associated with CVD
Blood	REAL TIME	FBC	Baseline, week 6, 12, 24 then every 24 weeks	Cardiac effects of anaemia
		Lipids (HDL, LDL, Cholesterol)		Dyslipidaemia a RF for CVD
		Biochemistry		Renal disease a RF for CVD
		Absolute CD4 count and percentage		Marker of HIV disease activity
		Immunophenotyping	Week 0, 48, 96	Evidence of immune activation and ongoing
	STORED samples	Viral Load	Baseline and week 96	Marker of HIV disease activity
		Inflammatory panel	Week 0, 48, 96	Markers of ongoing inflammation
		Disordered thrombogenesis		Evidence of disordered haemostasis
		Vascular injury panel		Evidence of vascular injury
Imaging	Carotid intima media thickness		Week 0, 48, 96	Structural arterial damage
	Pulse Wave Velocity			Arterial stiffness

RF, risk factor; CVD, cardiovascular disease; HDL, high density lipoprotein; LDL, low density lipoprotein. Inflammatory panel: (IL-1Ra, CRP, TNFa, IL-10, IL-6, IL-8), Disordered thrombogenesis: (DD, TM, TF), Vascular injury panel: (MCP-1, angiopoietin 1+2, E-selectin, P-selectin, ICAM-3, SAA, ICAM-1 VCAM-1, VEGF)

4.2 Cardiovascular skills training

A physician-sonographer were selected from both sites as well as a nurse from JCRC, Kampala participated in 2 weeks intense training with the Vascular Physiology laboratory team at University College London (UCL), Figure 4-1. Following an overview of the principles of IMT and PWV each medical practitioner practiced daily on different subjects until they were deemed competent to perform the recordings unaided. I performed further in country training during which an additional doctor and 4 nurses were trained using the same training methods - Figure 4-2. Overall 8 medical practitioners (3 doctors and 5 nurses) completed the basic training, 4 at JCRC and 4 at UTH.

Figure 4-1. Cardiovascular training taking place in the Vascular Physiology Laboratory, UCL.

Once training had been completed, each medical practitioner took part in a validation study to assess the outcome of the training. This ensured quality in the reproducibility and accuracy of the measurements performed by each individual. Healthy adult volunteers were recruited at each site from colleagues, friends and family. The volunteers were counselled and verbal consent obtained. All volunteers were scanned in the morning to minimise fluctuations in environmental temperature which can affect PWV (See Section 3.2) and where possible had fasted for a minimum of 3 hours prior to the recording being performed. Volunteers were examined by all 4 medical practitioners at either UTH or JCRC and the examinations repeated at least 24 hours after the first examination to allow comparison of intra and inter operator variability. The validation study continued until all medical practitioners had performed reproducible cIMT and PWV recordings on at least 8 of the volunteers twice. All recording were transferred electronically to the UK and were read by myself with support from an established sonographer where needed.

Figure 4-2. Cardiovascular training taking place at UTH, Lusaka.

4.2.1 Validation of cIMT and PWV

Previous studies have considered intra-observer variability of IMT between 2.4% to 10.6% and inter-observer variability of between 3.1% to 18.3% to be acceptable [322]. For an IMT scan to be accepted all criteria listed in section 3.3 had to be met and all readings (end diastolic diameter, systolic diameter and end-diastolic IMT) from both sides had to be within 0.1mm of each other for the scan to be included in the study. An example of a rejected scan is given in Figure 4-3.

For PWV readings all measurements had to be within 0.5m/second of each other. The lengths measured (carotid-notch, notch-femoral, carotid-femoral) were also checked to ensure accuracy and reproducibility between medical practitioners.

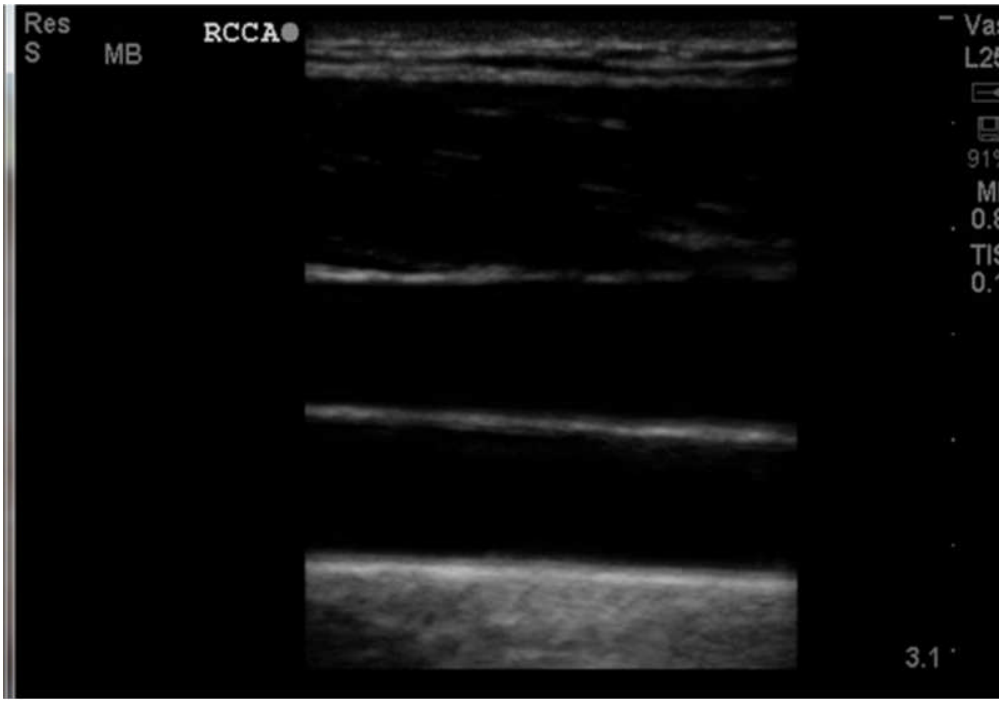


Figure 4-3. An example of a poor quality IMT scan

The artery is not positioned in the middle of the screen, the carotid bulb is not visible, the artery is not horizontal and the cut throught the artery is not at 90^{o} so the near wall IMT is not clearly defined.

In Zambia 4 medical practitioners (A, B, C and D) performed 117 IMT scans and 98 PWV recordings on 15 subjects during the period 15th June 2010 – 28th October 2010. In Uganda 3 medical practitioners (E, F, and G) performed 81 IMT scans and 75 PWV recordings on 14 subjects during the period 31st May 2010 – 11th November 2010. A 4th Ugandan medical practitioner, H, was unable to complete a sufficient of number scans; results were excluded from the statistical analysis.

Only the scans that fulfilled the criteria for a satisfactory scan were used in the following calculations.

Table 4-3. Summary of total IMT and PWV recordings performed during the validation study.

		IMT		PWV	
		Total number of scans performed	Number (%) of acceptable scans	Total number of scans performed	Number (%) of acceptable scans
ZAMBIA	A	22	17 (77)	15	15 (100)
	B	29	21 (71)	26	25 (96)
	C	31	16 (50)	27	22 (82)
	D	35	17 (49)	30	28 (93)
UGANDA	E	29	27 (93)	28	26 (93)
	F	31	21 (68)	29	27 (93)
	G	21	13 (62)	18	18 (100)

4.2.2 Intra-operator variability

To compare medical practitioners' personal variation when examining the same volunteer on subsequent days, the co-efficient of variation and a paired t-test to compare the mean of IMT and PWV were calculated. The results from volunteers who were only scanned successfully once were excluded from this analysis.

Table 4-4. Analysis of intra-operator variability.

Difference between scans performed on the same volunteers by the same medical practitioners (A – G) at least 24 hours apart.

		IMT			PWV			LENGTH	
		Number of volunteers*	CV	p-value	Number of volunteers*	CV	p-value	CV	p-value
ZAMBIAN MEDICAL PRACTICTIONERS	A	8	3.8%	0.80	7	4.1%	0.31	4.0%	0.49
	B	10	6.8%	0.64	12	7.1%	0.53	5.1%	0.88
	C	7	11.1%	0.11	10	7.5%	0.11	4.4%	0.69
	D	7	9.6%	0.31	13	8.1%	0.31	4.0%	0.72
UGANDAN MEDICAL PRACTICTIONERS	E	12	4.6%	0.48	12	5.6%	0.97	2.6%	0.31
	F	9	6.1%	0.57	12	7.5%	0.43	4.0%	0.54
	G	6	3.6%	**0.02**	9	7.1%	0.59	4.7%	0.86

*CV = co-efficient of variation; * to be included in this analysis a volunteer had to be successfully scanned twice by the same medical practitioner*

The coefficient of variation (CV) should be as small as possible and for this study a cut off of <10% was set as acceptable. Medical practitioners A, B, E, F and G had satisfactory intra-operator variability. Medical practitioner C failed to reach a satisfactory level of reproducibility for cIMT (CV = 11.1%). The proportion of acceptable scans for medical practitioners C and D were under 50%, which was deemed unacceptable. All medical practitioners had satisfactory intra-operator variability when performing PWV measurements (CV 4.1 – 8.1%)

Looking at the intra-operator variability in IMT the p-values for the co-efficient of variance for all medical practitioners were non-significant ($p>0.1$), with the exception of medical practitioner G ($p=0.02$). The null hypothesis was that the average difference between first and second measurements was zero; when the raw data for medical practitioner G was analysed in five out of the six cases the second IMT measurement was marginally higher than the first, so the significant p value was reflecting this trend. All the other parameters measured were satisfactory for medical practitioner G and as such this p value was interpreted with caution as it was not believed that there was a statistical significance between IMT readings taken on subsequent days.

4.2.3 Inter-operator variability for cIMT

Table 4-5 lists the co-efficient of variance and p values for the differences in the mean value obtained at the first IMT examination of the same volunteers by each medical practitioner.

Table 4-5. Results of the analysis of inter-operator variability

		IMT					
		CV (%)			p-value		
ZAMBIA		**A**	**B**	**C**	**A**	**B**	**C**
	B	13.0			0.29		
	C	8.6	10.5		0.68	0.90	
	D	6.1	9.3	6.7	0.33	0.22	0.64
UGANDA		**E**	**F**		**E**	**F**	
	F	6.8			0.50		
	G	3.8	5.4		0.90	0.52	

Medical practitioners “A” in Zambia and “E” in Uganda were used as a baseline to which the other medical practitioners results were compared using a mixed-effects restricted maximum likelihood (REML) model. The REML approach is a form of maximum likelihood estimation that does not base estimates on a maximum likelihood fit of all the information, but instead uses a likelihood function calculated from a transformed set of data, so that nuisance parameters have no effect.

The regression coefficients indicated that medical practitioner A slightly under measured cIMT compared to B, C and D and that medical practitioner E slightly under/over measured cIMT compared to F/G. However with an overall p value of 0.30 and with all the confidence intervals containing 0, no overall significant difference

between the IMT results was seen between different medical practitioners. An intra-class correlation (ICC) calculation to assess overall reproducibility between all the medical practitioners was calculated whereby a result >0.80 is deemed very good. The ICC was 0.94 for the Zambian team and 0.83 for the Ugandan team,

4.2.4 Inter-operator variability for PWV

Table 4-6 details the CV / p values reflecting the difference between PWV results when the same volunteer was scanned by different medical practitioners. Unacceptable differences in the results obtained when A and C measured PWV in the same volunteer were seen (p=0.04). An ANOVA calculation suggested that for the Zambian medical practitioners there was a trend towards a difference in the PWV measured between medical practitioners (p=0.05) with statistically significant larger PWV values obtained by C (p=0.01) and D (p=0.03) compared to A. For the Ugandan medical practitioners there was no statistical difference in the PWV measurements (p>0.50). For the length measurements again as expected a difference was seen between volunteers (p<0.001) but the variation between medical practitioners was not significant (p=0.40 / 0.84).

The ICC for PWV at both sites (Zambia/Uganda) (0.72/0.68) and length (0.77/0.77) were all deemed to be highly acceptable.

Table 4-6. Results of the analysis of inter-operator variability for PWV measurements.

		PWV						PWV length					
		CV (%)			p-value			CV (%)			p-value		
		A	B	C	A	B	C	A	B	C	A	B	C
ZAMBIA	B	5.8			0.48			6.2			0.18		
	C	7.4	8.8		**0.04**	0.35		4.1	3.6		0.25	0.49	
	D	7.3	7.1	7.9	0.55	0.97	0.12	3.9	4.8	4.5	0.65	0.61	0.93
UGANDA		E	F		E	F		E	F		E	F	
	F	6.9			0.77			4.5			0.20		
	G	8.2	8.1		0.86	0.95		3.6	5.6		0.72	0.57	

CV, co-efficient of variance.

In summary, medical practitioners A, B, E, F and G were all deemed competent to proceed with performing scans on the patients. Medical practitioners C and D were asked to undertake further practice in the techniques and to repeat the validation study. Unfortunately this was not possible in the time available before the CHAPAS 3 trial started recruiting and they were therefore unable to scan any of the patients.

Figure 4-4. The JCRC team.

From left to right Dr Victor Musiime, Mrs Florence Odongo, Dr Grace Mirembe, Mrs Priscilla Wavamunno.

4.2.5 Ongoing validation

Ongoing training and validation was arranged during the 3 years of CHAPAS 3 data collection. Yearly refresher courses and repeat mini-validation studies were carried out with satisfactory results obtained by all medical practitioners throughout (data not shown). Fortunately only one medical practitioner left during the trial and the remaining team had the capacity to continue the scans so no new personnel had to be trained.

4.2.6 Minimising confounders and blinding

To maximise consistency, where possible sequential scans on each patient were performed by the same practitioner. Given the small number of medical practitioners at each site this was usually possible. As the scans were being performed by the children's own medical team it was impossible to blind the medical practitioners performing the scan to the patient's ART randomisation arm, although where possible the scans were performed before the case notes were reviewed. All scans were electronically transferred and read by myself; I was blind to HIV status and treatment arm. Overall 5% of scans were checked by an experienced sonographer to ensure consistency in interpretation.

4.3 Discussion

The CHAPAS 3 trial provided a structure to intensively study cardiovascular structure and arterial stiffness in a large group of HIV infected children in an African setting. If successful this would be the largest study of HIV infected children worldwide and the only longitudinal study to date in an African setting. Compared to other studies the population to be studied would be unique with a large ART naïve group (208 children) and the ART experienced children were all stable on the same first line ART regimen. Different individual drugs, such as protease inhibitors, have been shown in other studies to have detrimental effects on IMT/PWV so by controlling for previous and current ART exposure results are easier to interpret. By studying very young children the impact of other cardiovascular risk factors such as obesity and smoking were minimised. By carefully selecting HIV uninfected controls from the same community differences in confounders such as co-infections, malnutrition and diet were limited.

I was able to devise a process for training and to demonstrate the reproducibility of medical practitioners measuring cIMT and PWV following an intensive training course. Medical practitioners who failed to reach the standard were prevented from scanning the study participants. Ongoing training and validation ensured that the standards remained high throughout. Methods were put in place to maintain consistency and blinding to group and randomisation arm.

Chapter 5 Results: Structural vascular changes in children with HIV

As discussed in chapter 2 HIV-infected adults have an increased incidence of cardiovascular disease whilst older children and adolescents have evidence of pre-clinical changes in the structure and arterial stiffness of their cardiovascular system. However, minimal data from children living in Africa is available. Detecting pre-clinical changes during childhood is important as underlying cardiovascular disease may be silent until a child reaches adulthood. Two methods of detecting pre-clinical changes, IMT and PWV have been described in chapter 3. These techniques were used to assess the cardiovascular structure and arterial stiffness of a large cohort of ART naïve and experienced HIV-infected children and age matched controls.

5.1 Methods

5.1.1 Recruitment

As described in chapter 3 children were recruited to the CHAPAS 3 trial. 208 HIV-infected ART naïve children were recruited (group 1) with 209 age matched HIV-uninfected children recruited as a control group. 74 HIV infected ART experienced children were also recruited (group 2) with 75 age matched HIV uninfected children as a second control group. Sites were asked to age match controls to within a year either side of the age of the CHAPAS 3 recruits at baseline. Controls were seen once; children in groups 1 and 2 were seen at 12 weekly intervals up until the end of the CHAPAS 3 trial, defined as when the last patient recruited reached week 96 of follow up. All children recruited to group 2 reached week 96 of follow up. 13 children in group 1 died and 16 were lost to follow up before week 96 - Figure 5-1.

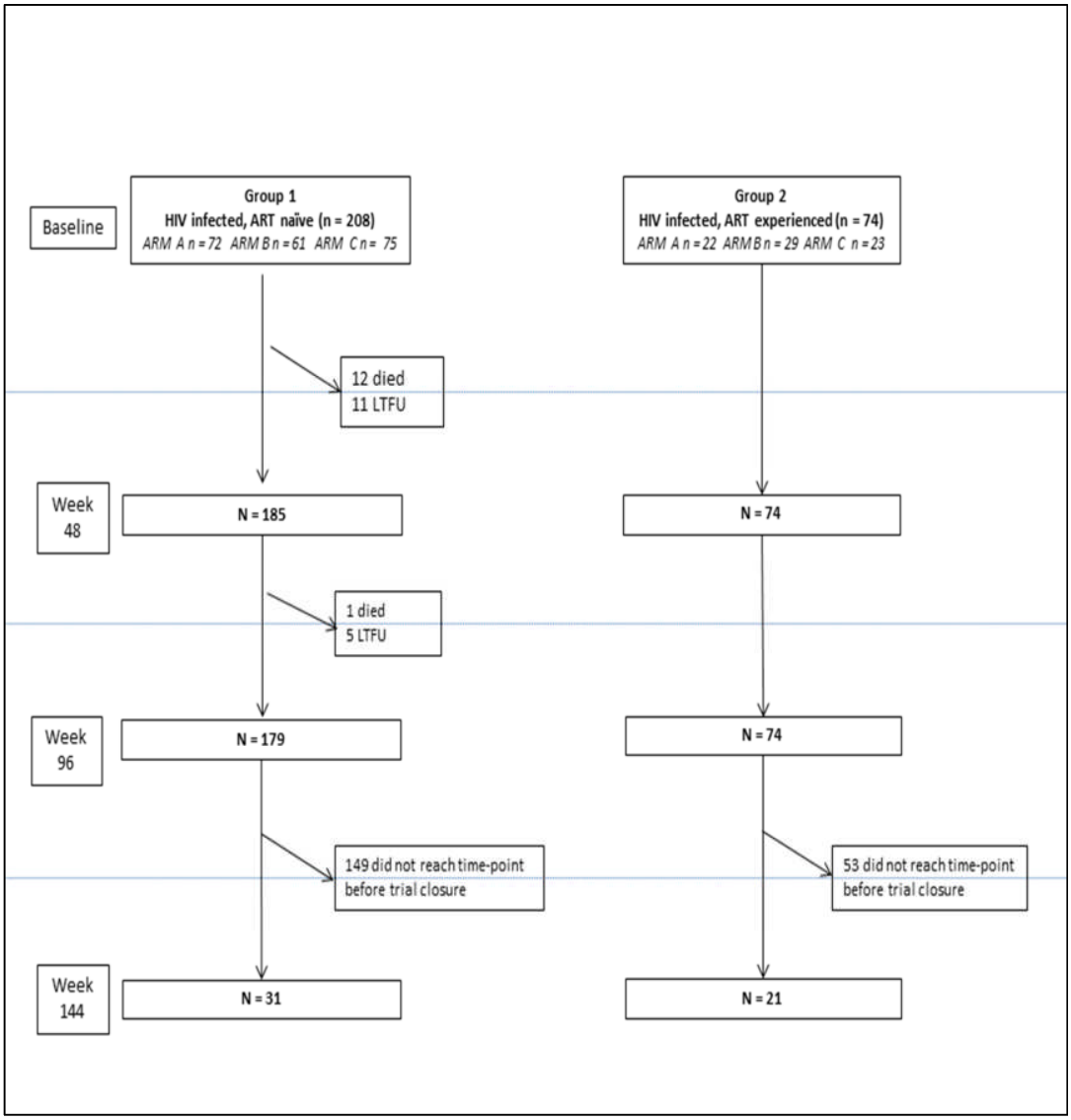


Figure 5-1. Flow diagram to show the outcome of children recruited to CHAPAS-3.

Arm A, stavudine; Arm B, zidovudine; Arm C, abacavir; LTFU, lost to follow up.

5.1.2 Baseline demographics

Clinical and immunological factors

Baseline demographic and clinical characteristics are listed in Table 5-1. All children were Black-African and had vertically acquired HIV. 208 were ART naïve (group 1) and these children were significantly younger (median age 2.9 years, range 0.3-13.6 years) than the 74 ART experienced (group 2) children (median age 6.9 years, range 5.1-12.3 years). The percentage of males was 52% and 54% in group 1 and 2 respectively. Group 1 and 2 were well matched to their control groups for sex (p=0.35/0.80) and age (p=0.21/0.60).

Not unsurprisingly between group 1 and the control group there were significant differences in CD4 count, CD4%, weight-for-age z-score, height-for-age z-score, BMI for age z score and CD4 z score (all p<0.001) with the HIV infected cases all significantly impaired. Group 2 and controls were well matched for CD4% (p=0.11) and BMI for age z score (p=0.90) but impairments were seen in the HIV infected children in weight for age z score (p=0.03) and height for age z score (p=0.01). Interestingly, despite CD4% being similar, CD4 z-scores and CD4 counts were significantly higher in group 2 (who had been on ART for a median of 3.9 years) compared to their age and sex matched controls (p=0.002 and p=0.004 respectively). Most ART naïve children had not had a CD4 count measured previously so the screening CD4 count was taken as their nadir. Of note the healthy control children were significantly lighter and shorter than UK children of an equivalent age (mean WAZ in control groups -0.8 / -0.9 and mean HAZ in control groups -1.2 / -1.3), if the control population was perfectly matched to the reference population the mean should be 0 and the standard deviation 1.

Cardiovascular parameters

Specifically looking at known cardiovascular risk factors no child was overweight / obese (defined as a BMI >85/95th percentile). Significantly less healthy (i.e. higher) diastolic blood pressure (p=0.002) and resting heart rate (p<0.001) were seen in ART naïve children in group 1 compared to their age matched controls.

Table 5-1. Demographic data and clinical parameters for all children recruited.

	HIV infected, ART naïve (Group 1) n = 208	HIV uninfected matched to group 1 n = 209	p value (1)	HIV infected, ART experienced (Group 2) n = 74	HIV uninfected matched to group 2 n = 75	p value (1)
Clinical variable						
Male	108 (52%)	99 (47%)	0.35	40 (54%)	39 (52%)	0.80
Age in yrs	2.9 (1.7 : 4.4)	3.0 (2.1 : 4.1)	0.21	6.9 (5.9 : 8.5)	6.7 (5.6 : 8.6)	0.60
Weight-for-age Z	-2.4 (-3.4, -1.1)	-0.8 (-1.6, -0.02)	**< 0.001**	-1.3 (-2, -0.5)	-0.9 (-1.9, -0.2)	**0.03**
Height-for-age Z	-2.6 (-3. 7 : -1.6)	-1.2 (-2.2 : -0.1)	**< 0.001**	-1.7 (-2.6 : -1.2)	-1.3 (-2.2 : -0.5)	**0.01**
BMI-for-age Z	-0.8 (-1.7 : 0.2)	-0.1 (-0.9 : 0.5)	**< 0.001**	-0.3 (-0.8 : 0.3)	-0.1 (-0.9 : 0.5)	0.90
Mean sBP (mmHg) (SD)	95 (9)	95 (12)	0.82	100 (8)	101 (8)	0.21
Mean dBP (mmHg) (SD)	59 (8)	56 (10)	**0.002**	60 (7)	59 (7)	0.21
Mean HR (mmHg) (SD)	110 (17)	98 (16)	**< 0.001**	91 (13)	91 (16)	0.92
HIV related variables						
Current WHO stage (%)	I/II 91 (44) III 97 (47) IV 20 (10)			I/II 62 (84) III 7 (10) IV 5 (7)		
Previous WHO stage III/IV (%)	119 (57)			46 (62)		
Baseline VL (copies/ml)	314,565 (134830 : 810820)			<50 copies / ml		
Duration on ART (yrs)				3.9 (2.9 : 4.3)		
Age started ART (yrs)				3.2 (1.8 : 5.0)		
Immunological variables						
CD4 count	823 (507 : 1244)	1335 (1012 : 1781)	**< 0.001**	1192 (893 : 1532)	974 (823 : 1175)	**0.004***
CD4 %	18 (11 : 23)	37 (32 : 41)	**< 0.001**	33 (27 : 39)	35 (31 : 42)	0.11
CD4 z score	-2.0 (-3.0 : -1.1)	-0.6 (-1.3 : 0.3)	**< 0.001**	0.1 (-0.7 : 0.8)	-0.6 (-1.1 : 0.02)	**0.002***
Lipids						
Total cholesterol (mmol/L)	3.1 (2.6 : 3.8)	3.6 (3.1 : 4.3)	**<0.001****	4.1 (3.6 : 4.5)	3.6 (3.1 : 4.1)	**0.001**
Triglycerides (mmol/L)	1.2 (0.9 : 1.8)	1.0 (0.7 : 1.5)	**< 0.001**	0.9 (0.7 : 1.0)	0.7 (0.5 : 1.1)	0.07
LDL (mmol/L)	1.7 (1.3 : 2.3)	2.0 (1.6 : 2.4)	**0.001****	2.1 (1.7 : 2.5)	1.8 (1.5 : 2.4)	0.09
HDL (mmol/L)	0.7 (0.5 : 0.8)	1.0 (0.9 : 1.5)	**< 0.001*****	1.4 (1.1 : 1.6)	1.3 (1.0 : 1.7)	0.21
Hypercholesterolemia >5.2mmol/L	5/187 (3%)	18/192 (9%)	**0.01****	7/66 (11%)	7/71 (7%)	0.56
Hypertriglyceridemia >1.7mmol/L	51/182 (28%)	35/190 (18%)	**0.02**	4/65 (6%)	5/71 (7%)	0.56
HyperLDL >3.4mmol/L	5/180 (3%)	8/192 (4%)	0.33	2/59 (3%)	2/71 (3%)	0.62
HypoHDL <0.9mmol/L	147/184 (80%)	52/192 (27%)	**< 0.001**	5/64 (8%)	14/71 (20%)	**0.04**
Minutes fasting prior to lipid sample	810 (286 : 900)	120 (69 : 310)	**< 0.001**	845 (796 : 930)	477 (164 : 1058)	0.19

*(1) Mann Whitney rank sum used for non parametric variables, two sample t-test used for parametric variables. Values are given as median and IQR unless otherwise stated. BMI, body mass index; dBP, diastolic blood pressure; HDL, high density lipoprotein; HR, heart rate; LDL, low-density lipoprotein; sBP, systolic blood pressure; VL, viral load. *CD4 and CD4 z scores higher in HIV infected, ART experienced children compared to controls ** cholesterol and LDL levels higher in controls compared to HIV infected ART naive children ***HDL levels higher (healthier) in HIV infected, ART experienced children compared to controls.*

Lipids

ART naïve children had significantly healthier (lower) total cholesterol and LDL-C both (p=<0.001) than controls but less healthy (lower) HDL-C and (higher) triglyceride levels (both p<0.001). In the ART experienced group total cholesterol profiles were significantly worse (higher) compared to controls (p=0.001), whilst HDL-C, triglyceride and LDL-C levels were not significantly different between HIV infected and uninfected children (p≥0.07). Using internationally recognised cut offs [323] the proportion of ART naïve/controls and ART experienced/controls with hypercholesterolemia was 3%/9% and 11%/7%; hypertriglyceridemia, 28%/18% and 6%/7%; hyperLDL-C 3%/4% and 3%/3%; hypoHDL-C 80%/27% and 8%/20%. Some of these results may reflect the fact that recruitment of the controls was opportunistic at times and hence it was difficult to ensure that the children had fasted for an adequate period of time; mean period of fasting in group 1 v controls was 810 v 120 minutes (p<0.001), in group 2 v controls 845 v 477 minutes (p=0.19). Whilst the international guidelines used did not specify an age range, it is also important to note that in this cohort a large proportion of children were aged less than two years for which normal ranges are less well defined. The proportion of children with hypertriglyceridemia and hypoHDL-C differed significantly between children aged less than two years and children aged over two years of age - Table 5-2.

Past medical history and viral load

Among group 1 and 2 57% / 62% had experienced WHO stage III/IV events. To be recruited to the study children in group 2 had to have virological suppression (assay <50 copies / ml) and have been on first line ART for at least 2 years, the median (range) of prior ART treatment was 3.9 (2 : 7.1) years. In group 1 1% had a baseline VL <1000 copies/ml; 2% 1000-9,999 copies/ml; 14% 10,000–99,999 copies/ml; 63% 100,000–999,999 copies / ml and 19% ≥1,000,000 copies / ml.

Table 5-2. Summary of lipid levels in the ART naive children and their controls looking at the effect of age under 2 years of age compared to greater than two years of age.

	< 2 years			> 2 years			Overall effect of age < 2 or > 2 years
	HIV infected, ART naïve (group 1)	HIV uninfected matched to group 1	p value	HIV infected, ART naïve (group 1)	HIV uninfected matched to group 1	p value	
Number of samples available	65	40		122	152		
Total cholesterol (mmol/L)	2.8 (2.3 : 3.6)	3.6* (3.0 : 4.8)	**<0.001**	3.3 (2.8 : 3.8)	3.6* (3.1 : 4.2)	**<0.001**	
Hypercholesterolaemia >5.2 mmol/L	1 (1.5%)	7 (18%)	**0.002**	4 (3.3%)	11 (7.2%)	0.15	p = 0.43
Triglycerides (mmol/L)	1.6 (1.2 : 2.4)	1.2 (0.9 : 1.8)	**0.03**	1.1 (0.9 : 1.5)	0.9 (0.6 : 1.3)	**<0.001**	
Hypertriglycercidemia > 1.7mmol/L	29 (46.8%)	13 (34.2%)	0.22	22 (18.3%)	22 (14.5%)	0.39	**p < 0.001**
LDL (mmol/L)	1.5 (1.0 : 2.0)	1.9 (1.4 : 2.3)	**0.01**	1.9 (1.4 : 2.4)	2.0 (1.6 : 2.4)	0.13	
HyperLDL >3.4 mmol/L	1 (1.6%)	2 (5%)	0.33	4 (3.4%)	6 (3.9%)	0.81	p = 0.72
HDL (mmol/L)	0.6 (0.4 : 0.7)	1.0 (0.9 : 1.6)	**<0.001**	0.7 (0.6 : 0.9)	1.1 (0.9 : 1.5)	**<0.001**	
HypoHDL <0.9mmol/L	58 (90.6%)	12 (30%)	**<0.001**	89 (74.2%)	40 (26.3)	**<0.001**	**p = 0.001**
Minutes fasting prior to sample	280 (122 : 840)	80 (34 : 150)	**0.001**	845 (740 : 925)	135 (79 : 361)	**<0.001**	

Absolute values compared between groups using rank sum and categorical values compared using chi-squared. Values are given as median and IQR unless otherwise stated. HDL, high density lipoprotein; HR, heart rate; LDL, low-density lipoprotein.
** cholesterol levels higher in controls*

5.2 CD4 count / percentages in HIV negative controls

As illustrated in Figure 5-2 the majority of the 284 HIV uninfected children recruited to the control study had a satisfactory absolute CD4 count / percentage, however 25 (9%) of the "healthy" HIV negative controls fulfilled absolute or percentage CD4 count immunological criteria for commencing ART according to WHO 2013 guidelines[1] although only five (2%) met criteria on both absolute CD4 count and CD4 percentage - Table 5-3. These children may have been suffering from infections such as Epstein Barr Virus (EBV) or cytomegalovirus (CMV) that provoke a lymphocytosis with a relative increase in CD8 and overall lowering of the relative CD4%. Normal ranges are based on values derived from the European Collaborative Study (ECS) [324]; whilst a substantial proportion of these children were of African origin no normal data exists for HIV-infected African children living in Africa. A provisional study looking at normal ranges in African HIV-infected infants has not found a difference from the values obtained from the ECS *(Helen Payne, personal communication).* In other cohorts of HIV uninfected African children ≥ 99% aged over three months of age have a CD4 count above the threshold for treatment initiation [325]. Maternal HIV status for the controls was not collected so some of these children may be HIV exposed but uninfected [326, 327]. All children recruited to the control study aged less than 18 months had a HIV DNA PCR performed and children over 18 months an HIV fourth generation antibody test. It is possible that these gave a false negative HIV result and in fact the child was HIV infected; increasingly early false negatives are being recognized especially if children were breastfed whilst their mothers were taking ART [328]. Finally the CD4 measurement was only performed once so may be falsely low if there was a sample processing error.

[1] WHO 2013 immunological guidelines for commencing ART: < 3years CD4 < 1000 / CD4% < 25%, 3 – 5 years CD4 < 750 / CD4 < 25%, >5 years CD4 <500

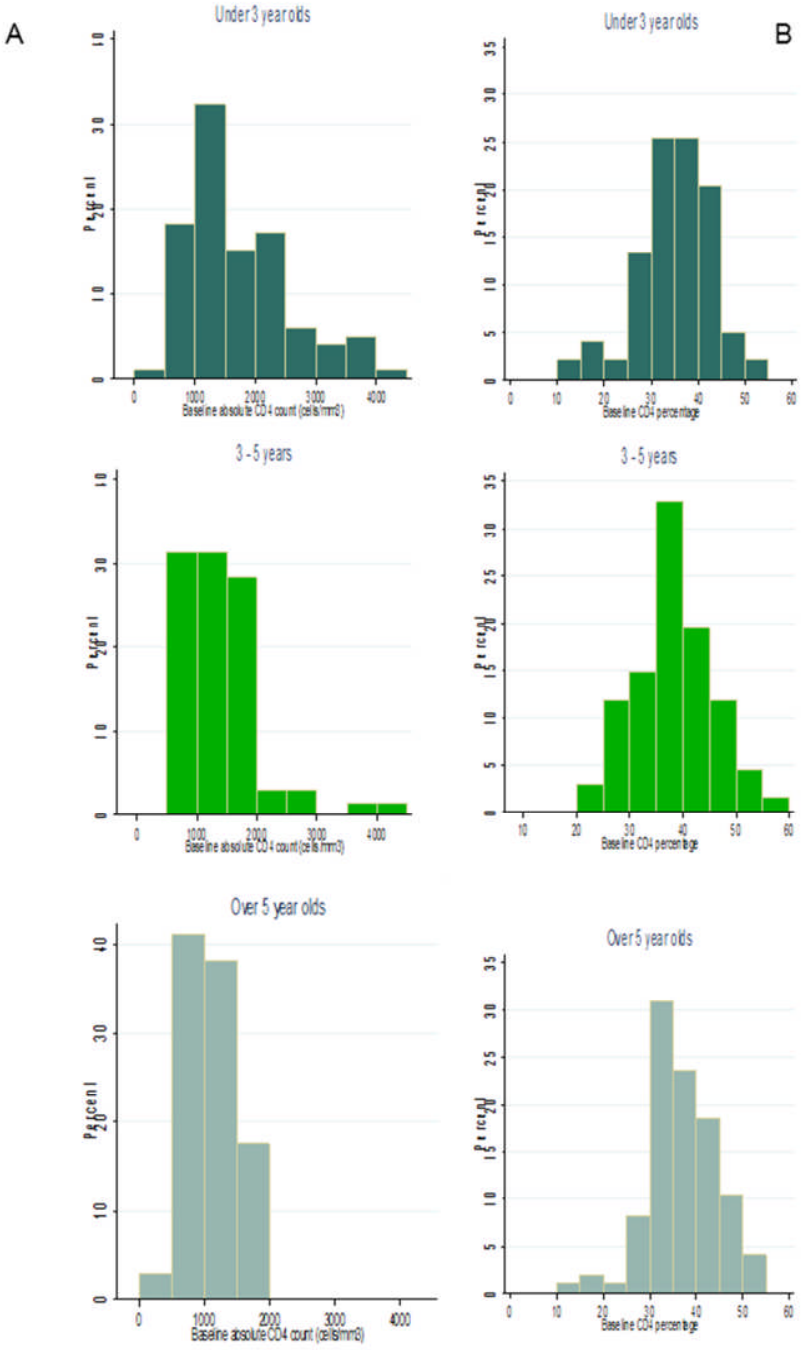


Figure 5-2. Distribution of CD4 count and absolute percentage in HIV uninfected controls by age band.

A) CD4 count and B) CD4 percentage

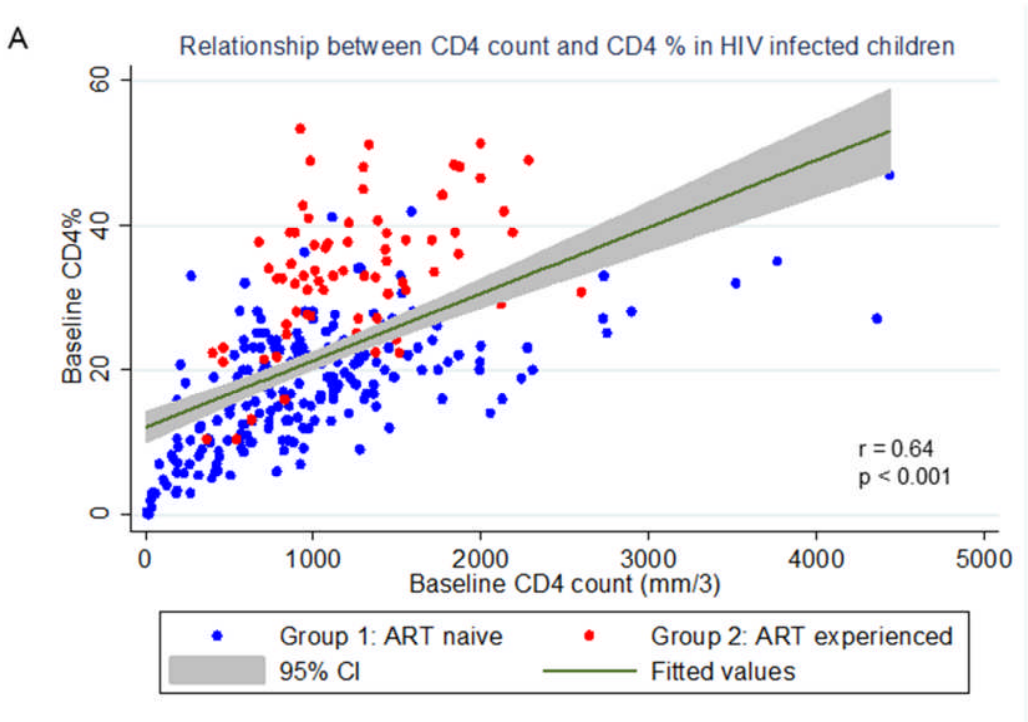
Table 5-3. HIV uninfected controls with low absolute CD4 count or percentage

ID	AGE (years)	Absolute CD4	CD4%	Total CD3	Lymphocytes (%)	CD3+ CD8- %	CD3+ CD8+ %	CD4:CD8	CD4 z score
K20007	0.7	1100	19	5929	46.6	31.1	48.8	0.6	-2
K10009	0.8	2000	22	9110	35	21.5	28.8	0.7	-0.8
K10020	1.5	856	15.5	5520	ND	ND	ND	ND	-2.2
C20040	2	970	12	7865	ND	ND	ND	ND	-1.7
F20048	2.1	1185	23	5224	29.7	37.7	40	0.9	-1.2
G20042	2.2	631	27	2305	65.3	70.2	20.5	3.4	-2.6
H20027	2.4	716	29	3426	38.2	65.4	15.9	4.1	-1.1
Y10029	2.6	2000	13.3	7300	ND	ND	ND	ND	0.2
S20030	2.6	743	25	2928	30.8	55.7	26.9	2.1	-2.2
W10016	2.7	681	26.5	2570	37.2	31.4	19.9	1.6	-2.3
Y10035	2.7	998	25	4000	ND	ND	ND	ND	-1.4
W20024	2.8	603	41	1484	11.6	44.7	28.3	1.6	-2.6
G10022	2.9	342	17.3	1980	16.7	20	53.1	0.4	-3.9
P10031	2.9	965	19.1	5060	ND	ND	ND	ND	-1.4
R10040	3.2	836	23.5	3560	40.6	35.6	36.6	1	-1.7
H20070	3.3	620	29	2110	27	42.7	45.7	0.9	-2.4
S20047	3.5	708	33	3288	38.7	44.5	30.5	1.5	-0.9
V20080	3.7	652	36	1824	24.6	56.6	40.9	1.4	-2.2
C10055	4	649	27.6	2350	37.5	20.9	34	0.6	-2.2
Y20096	4.2	622	30	2088	10.3	45.5	32.2	1.4	-2.3
A10054	4.3	623	27.9	2330	31.4	30.7	27.4	1.1	-2.2
G10151	4.7	603	21.6	2790	ND	ND	ND	ND	-2.3
P10075	5.6	380	27.3	1390	ND	ND	ND	ND	-3.7
Y10102	6.9	274	16.6	1650	ND	ND	ND	ND	-5
P20138	11.3	474	34	1397	21.9	50.7	41	1.2	-3

Shading indicates values that are below the recommended WHO threshold for commencing ART.

5.3 The relationship between CD4 count and percentage in HIV infected and uninfected children

As illustrated in Figure 5-3 a strong relationship between absolute CD4 and CD4 percentage in HIV infected children (groups 1 and 2) was demonstrated. This correlation was still seen at CD4 counts <500 mm^3 (Spearman's correlation coefficient, rho 0.64/0.45, both p<0.001). A weaker correlation was seen in the control groups (Spearman's correlation coefficient - rho 0.38/0.29 p<0.001/=0.02) although this may have been as the data was observed over a narrower range.



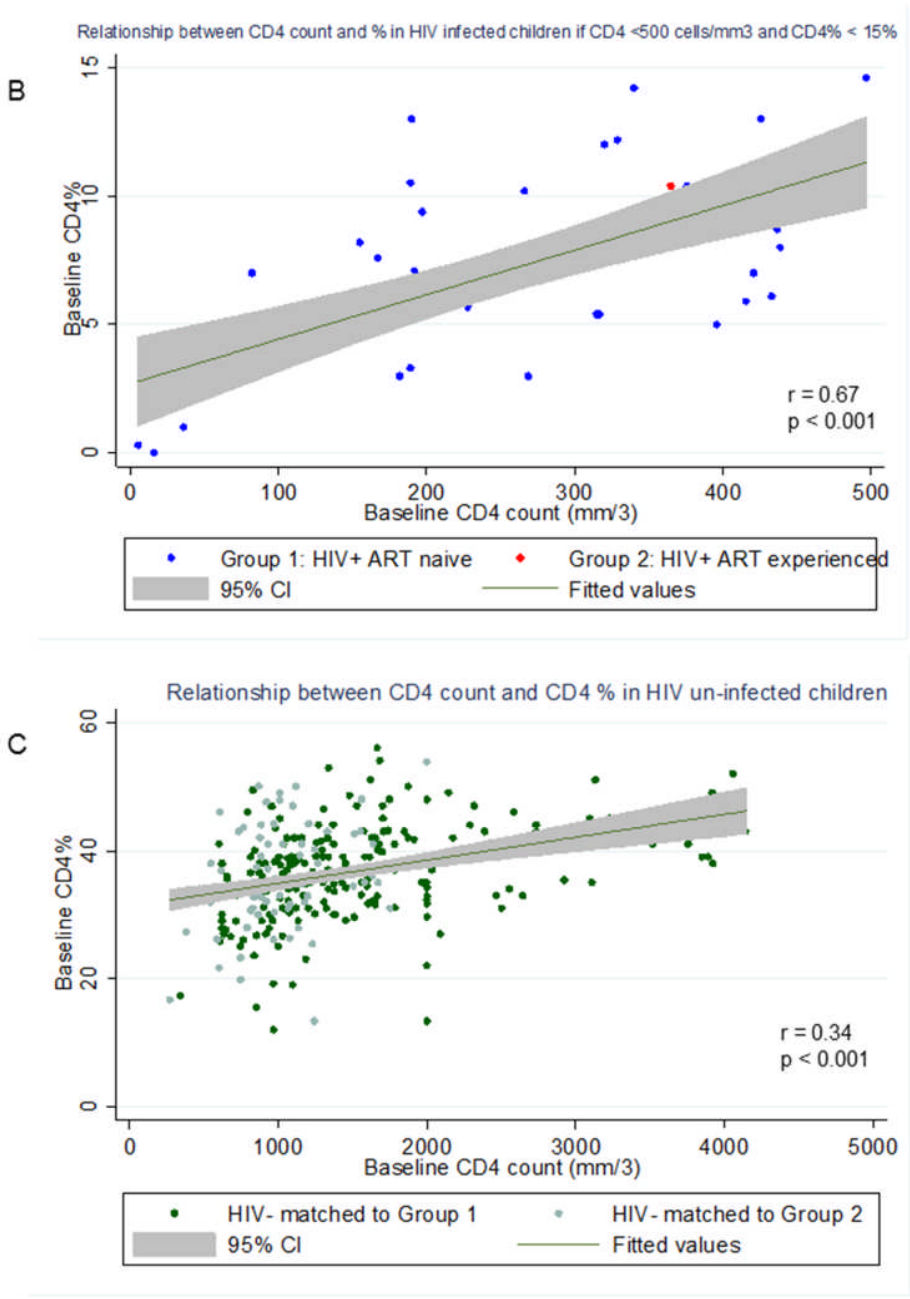


Figure 5-3. Scatter plots to show relationship between absolute CD4 and CD4 percentage.

A) HIV infected B) HIV infected, low CD4 and CD4% only C) HIV uninfected controls. Spearman correlation used.

5.4 Cardiovascular Results 1 – Intimal Medial Thickness (IMT)

5.4.1 Missing baseline IMT by site and group

Technical difficulties and uncooperative children meant some recruits could not be scanned and this tended to be the younger children -Table 5-4. Comparing the median age of children in groups 1 and 2 with their control groups respectively using a Wilcoxon rank sum test showed that at UTH, children with IMT results in group 1 were significantly older (median age 6.4 years) than their control group with IMT results (median age 4.1 years) (p=0.001). This can be explained as the controls were recruited later in the study when the clinical teams were more confident in scanning smaller children and technically they were able to perform high quality scans quickly - so more scans were performed on younger control children that were of acceptable quality. No difference in age of children with IMT results was seen between Group 2 and controls at UTH (p=0.78) nor at JCRC between Groups 1 and controls (p=0.46) or group 2 and controls (p=0.65). Combining all children recruited from both sites no difference in age was seen between Group 1 and controls (p=0.54) nor Group 2 and controls (p=0.61). Thus comparisons of HIV infected versus controls cannot be confounded by age as a whole; however any subsequent analyses which adjust for site will also be adjusted for age.

Table 5-4. Effect of missing baseline IMT scans.

	HIV+ ART naïve (group 1)	HIV- controls matched to group 1	HIV+ ART experienced (group 2)	HIV- controls matched to group 2
UTH				
Total recruited	89	89	52	52
Missing baseline IMT (% total)	50 (56%)	30 (34%)	1 (2%)	8 (15%)
Age	2.9 (1.8 : 6.1)	3.4 (2.1 : 5.6)	7.0 (5.8 : 8.2)	6.9 (5.6 : 8.3)
Age with a baseline IMT	6.4 (4.4 : 9.6)	4.1 (2.8 : 7.0)	7.0 (5.8 : 8.5)	6.9 (5.6 : 8.5)
Age with baseline IMT missing	1.8 (1.2 : 2.5)	2.5 (1.0 : 3.0)	5.5	6.2 (5.0 : 8.2)
JCRC				
Total recruited	119	120	22	23
Missing baseline IMT (% total)	13 (11%)	12 (10%)	1 (5%)	0
Age	2.8 (1.6 : 4.0)	2.9 (2.1 : 4.1)	6.5 (5.9 : 9.9)	6.4 (5.5 : 9.1)
Age with a baseline IMT	3.1 (1.8 : 4.2)	3.2 (2.1 : 4.2)	6.5 (6.0 : 9.9)	6.4 (5.5 : 9.1)
Age with baseline IMT missing	1.5 (1.1 : 2.2)	2.4 (1.7 : 3.0)	5.2	NA

5.4.2 Baseline Intimal Medial Thickness (IMT)

As summarised in Table 5-5 and illustrated in Figure 5-4 baseline IMT was significantly thicker in group 1 versus controls (p<0.001) with a similar trend for the smaller numbers in group 2 versus the group 2 control group (p=0.09). Results were unchanged after excluding 15 and 16 scans performed 2–12 weeks after enrolment in groups 1 and 2 respectively (p<0.001 / =0.09). There was also no evidence of a trend towards a difference in IMT with increasing time after baseline when the scan was performed (p=0.98/0.43). Therefore all scans (baseline and post baseline up to a maximum of 12 weeks) were included in further analyses.

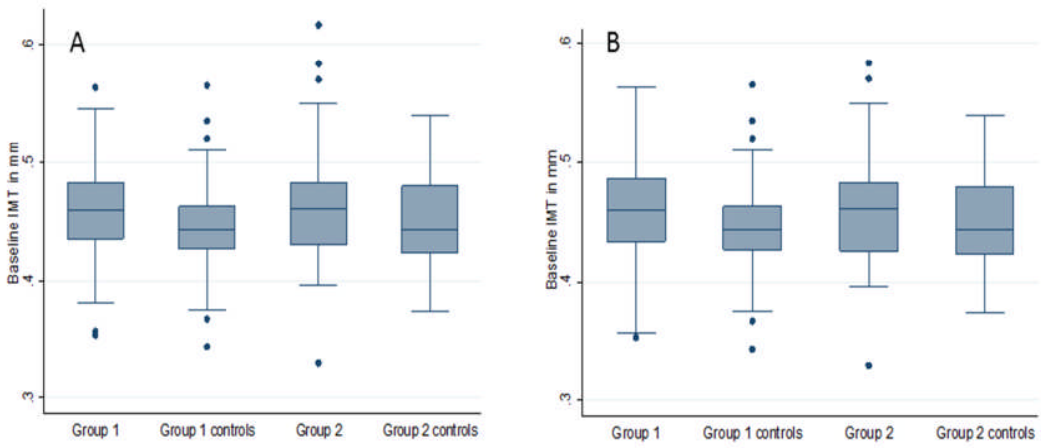


Figure 5-4. Box plot of baseline IMT by group (median and IQR).

A) Results when all baseline scans are included in the analysis; B) Results when IMT scans performed after week 0 are excluded.

Table 5-5. Summary of baseline IMT results by group

	HIV+ ART naïve group 1	HIV- controls matched to Group 1	HIV+ ART experienced group 2	HIV- controls matched to Group 2
Total number in group	208	209	74	75
Overall mean (SD)	0.45 (0.38)			
Median (IQR)	0.45 (0.43 – 0.48)			
Number IMT scans performed	145	167	72	67
Mean in mm (SD)	0.46 (0.04)	0.44 (0.04)	0.46 (0.05)	0.45 (0.04)
Range	0.35 : 0.56	0.34 : 0.57	0.33 : 0.62	0.37 : 0.54
Number performed after week 0 (% total)	15		16	
	Wk 2 9 (6%)		Wk 2 4 (6%)	
	Wk 6 4 (3%)		Wk 6 8 (11%)	
	Wk 12 2 (1%)		Wk 12 4 (6%)	
Missing (% total)	63 (30%)	42 (20%)	2 (3%)	8 (11%)
Too young (% missing)	53 (84%)	30 (71%)		
Not done	6 (10%)	2 (5%)	2 (100%)	1 (13%)
Unanalysable	2 (3%)	5 (12%)		7 (87%)
Missing*	2 (3%)	5 (12%)		

* missing scans – these were performed but accidently deleted prior to data transfer

5.5 Impact of variables on baseline IMT

5.5.1 Age

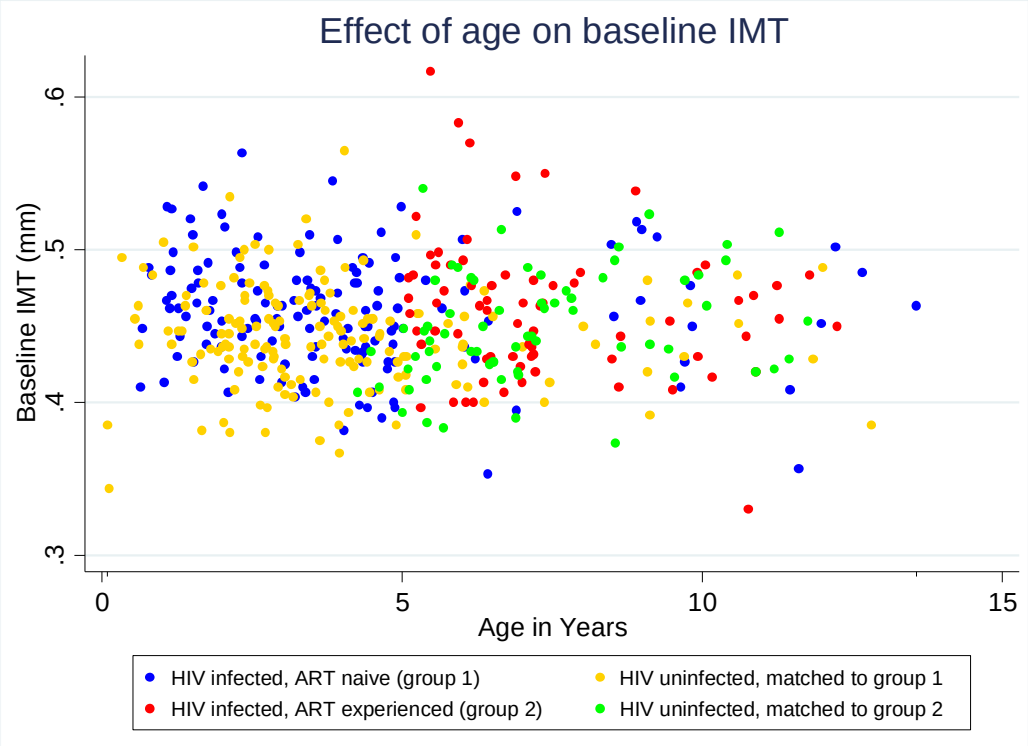


Figure 5-5. Scatter plot to show effect of age on baseline IMT

Comparing group 1 (ART naïve) and their control group in a full interaction model, allowing the relationship between baseline IMT and age to vary by HIV infection, estimates were that in the ART-naïve children baseline IMT was (-)0.001 lower for every year older (95% CI -0.004 : +0.001) (p=0.20), and in the controls baseline IMT was also (-)0.001 lower for every year older (95% CI -0.003 : +0.001) (p=0.39). There was no evidence that the impact of age at baseline varied by HIV status (interaction p=0.80). Fitting the same relationship between age and baseline IMT in both groups there was still no evidence of a relationship between baseline IMT and age overall – baseline IMT was (-)0.001 lower per year older (95% CI -0.003 ; +0.000) (p=0.13). The

strong association between baseline IMT and HIV status persisted (IMT being (-)0.016mm lower in controls than HIV-infected children (95% CI -0.024 : -0.008) p<0.001).

In contrast in the ART experienced group, there was evidence that the impact of age on baseline IMT varied by HIV status (p=0.01). In HIV infected ART experienced children baseline IMT was (-)0.005mm lower per year older (95% CI -0.010 : +0.000) (p=0.06). In controls, baseline IMT was (+)0.005 higher per year older (95% CI -0.000 : +0.010) (p=0.06).

Normal values of IMT in young children are not available. To attempt to calculate "normal range" IMT was plotted against age in all 234 HIV uninfected controls, Figure 5-6. Point estimate suggests that IMT is 0.001 mm higher per additional year of age but there is no formal evidence for this (95% CI -0.001 : +0.002) (p= 0.40).

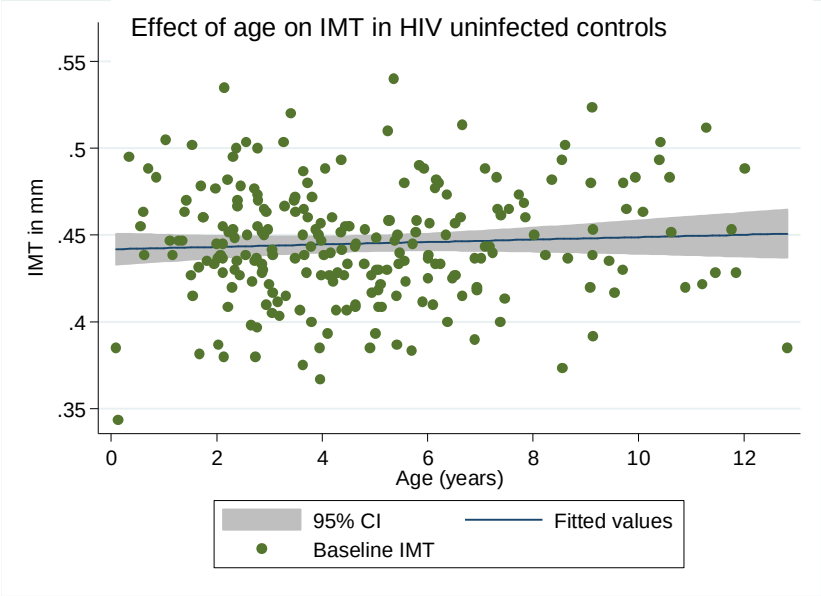
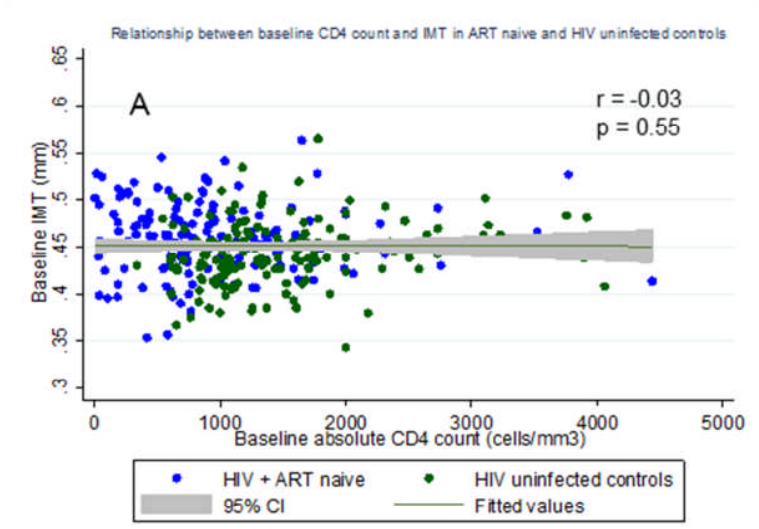


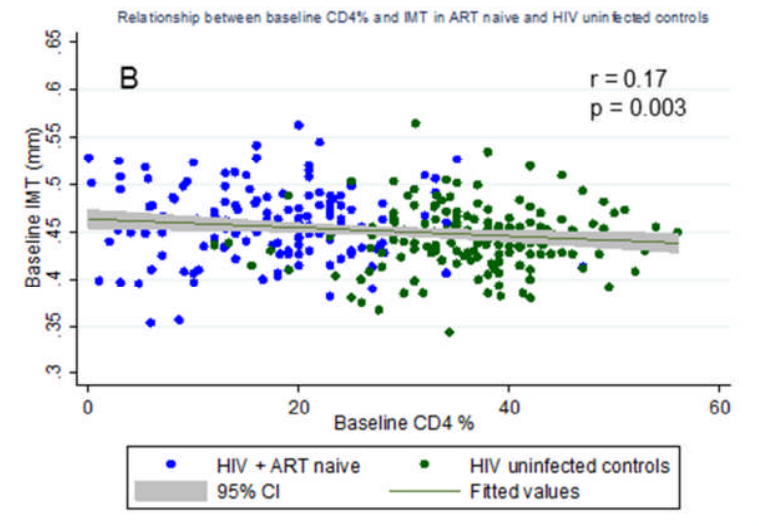
Figure 5-6. Scatter plot to illustrate effect of age on IMT in HIV uninfected controls.

No significant impact of age on IMT (p=0.40) was seen.

5.5.2 Impact of CD4 parameters on baseline IMT

CD4 counts were truncated at the 99th centile to avoid very high values having undue influence in regression models. A significant association between CD4% and baseline IMT was seen in ART naïve (group 1) children and controls (rho=0.17, p=0.003) (not adjusting for other factors). No significant association was seen between CD4 count and baseline IMT in ART naïve/controls, nor between CD4 count or percentage and baseline IMT in ART experienced/controls - Figure 5-7.





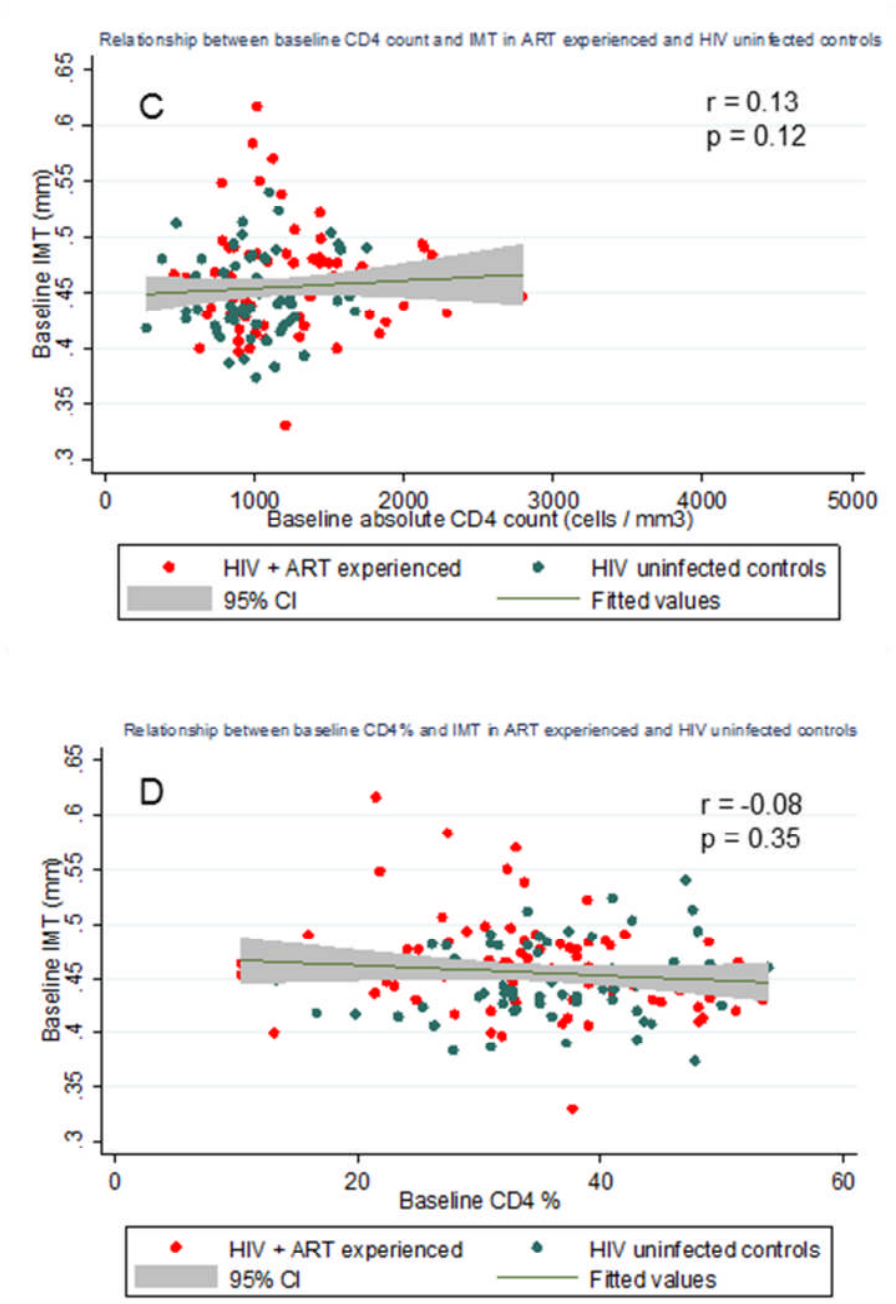


Figure 5-7. Scatter plots to demonstrate the relationship and Spearman correlation between baseline IMT and CD4 count / CD4%

A) CD4 count in ART naïve patients (group 1) and controls, B) CD4 percentage in ART naïve patients and controls C) CD4 count in ART experienced patients (group 2) and controls and D) CD4 percentage in ART experienced patients and controls.

5.5.3 Impact of other baseline variables on baseline IMT

A bivariable main effects and interaction model (allowing a different effect of each factor depending on whether the child was HIV-infected or a control) were used to screen the effects of other baseline variables on baseline IMT, Table 5-6. Variables such as baseline urea and age started ART were not included in this model as these were not available for the control group but were included in later models that assessed change over 96 weeks in the HIV-infected children. Any variable or interaction which had $p<0.2$ in the main effects or interaction model was included in a multivariable model from which a final multivariable model was identified using backwards elimination (exit $p=0.05$). Case/control status, age and site of recruitment were included in all models regardless of significance as the key exposure of interest (case vs control) or because they were factors a priori considered to have potential influence. After a model had been identified by backwards elimination, each rejected main effect and interaction term was checked for additional prognostic importance given this model, and any factors with $p<0.05$ retained.

Table 5-6. Results of a bivariate analysis, using a main effects and interaction model to screen for the effects of variables on baseline IMT.

	ART naïve versus controls		ART experienced versus controls	
	p value for main effect adjusted for group	p value for interaction by HIV status	p value for main effect adjusted for group	p value for interaction by HIV status
Clinical variable				
Age in years	**0.13**	0.80	0.98	**0.01**
Sex	**0.08**	0.30	**0.06**	0.73
Site	**0.04**	0.28	0.72	**0.11**
Weight-for-age Z	**0.12**	0.50	**0.08**	**0.03**
Height-for-age Z	**0.19**	0.44	0.80	0.23
BMI-for-age Z	0.40	**0.03**	**0.001**	**0.17**
Mean sBP	0.67	0.43	**0.07**	0.26
Mean dBP	0.27	0.69	0.35	**0.11**
Mean HR	0.65	**0.06**	0.55	0.59
HIV related variables				
Current WHO	0.69	NA	**0.05**	NA
Baseline log10 VL	0.28	NA	NA	NA
Duration on ART	NA	NA	0.25	NA
Immunological variable				
CD4	**0.15**	**0.16**	0.67	0.51
CD4%	0.96	0.47	0.35	**0.03**
CD4 z score	0.85	**0.06**	0.60	0.52
Lipids				
Total cholesterol	**0.01**	0.21	0.32	0.34
Triglycerides	0.39	**0.14**	**0.17**	0.50
LDL	0.31	**0.07**	0.37	0.33
HDL	**0.02**	0.98	0.70	**0.08**

Controls were classed as WHO stage 1, viral load = 100 and for those matched to group 2 (ART experienced) time on ART as 2 years. Main effects model included HIV status and factor of interest. Interaction model includes main effects model and interaction between HIV status and factor of interest. Absolute CD4 count and CD4 % were truncated at 99th centile. BMI, body mass index; dBP, diastolic blood pressure; HDL, high density lipoprotein; HR, heart rate; LDL, low-density lipoprotein; NA, not applicable; sBP, systolic blood pressure; VL, viral load.

The final multivariable model in ART naïve (group 1) v controls after backward elimination (Table 5-7) found that total cholesterol was inversely related to baseline IMT (IMT -0.006mm lower for every 1 mmol/L higher p=0.02). Age at baseline was not significantly related to baseline IMT after adjusting for total cholesterol (p=0.33). Despite the strong effect of total cholesterol, a residual effect of being an HIV infected case vs uninfected control remained, with IMT 0.01mm higher in cases after adjustment (p=0.02). After adjusting for total cholesterol and HIV status no significant effect of recruitment site persisted (p=0.11).

Table 5-7. Final multivariable models for baseline IMT

	Estimate of impact on IMT (mm)	95% CI	p value
Naïve & controls			
Mean at reference category	0.481	(0.464 : 0.498)	<0.001
Total cholesterol (per 1 mmol/L higher)	-0.006	(-0.010 : - 0.001)	**0.02**
HIV-infected case vs uninfected control	0.010	(0.001 : 0.018)	**0.02**
Site (UTH vs JCRC)	-0.008	(-0.019 : 0.002)	0.11
Age (per 1 year older)	-0.001	(-0.003 : 0.001)	0.33
Experienced & controls			
Mean at reference category	0.469	(0.438 : 0.500)	<0.001
HIV-infected case vs uninfected control	0.033	(0.012 : 0.054)	**0.03**
Weight-for-age in cases (per 1 unit higher)	0.014	(0.002 : 0.030)	**0.02**
Weight-for-age in controls (per 1 unit higher)	0.000	(- 0.008 : 0.010)	0.92
Site (UTH vs JCRC)	0.002	(-0.013 : 0.017)	0.97
Age (per 1 year older)	0.001	(-0.002 : 0.005)	0.44

Reference category = age 0 and total cholesterol 0. IMT, Intimal Medial Thickness; JCRC, Joint Clinical Research Centre, Kampala; UTH, University Teaching Hospital, Lusaka;

The surprising and somewhat counterintuitive finding that a higher cholesterol was significantly associated with a lower (healthier) IMT was looked at in more detail. The graphs displayed in Figure 5-8 confirm that the same direction of effect is seen when just total cholesterol is considered, so this finding is not due to over correcting in the multivariable model.

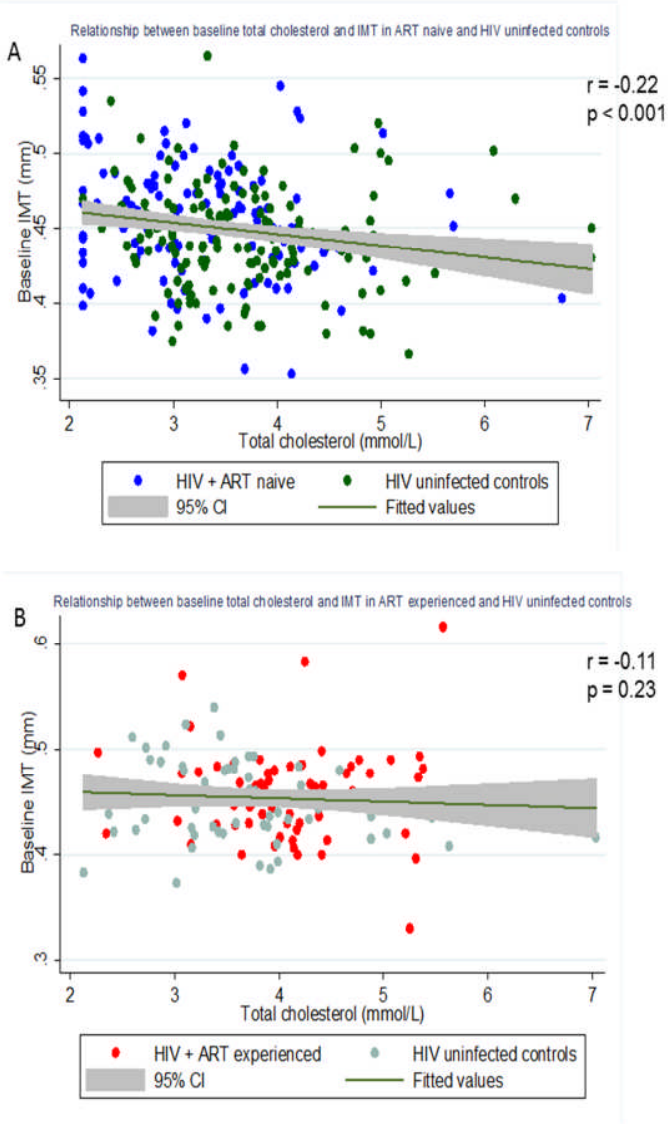


Figure 5-8. Relationship between baseline IMT and cholesterol.

The final multivariable model in ART experienced (group 2) v controls after backward elimination (Table 5-7) included weight-for-age which had effects that differed significantly between HIV-infected cases and uninfected controls (interaction p=0.04). In HIV-infected cases, IMT was +0.014mm higher per unit higher weight for age (95%CI (+0.002 : +0.030), p=0.002); but there was no association between weight-for-age and IMT in HIV-uninfected controls (p=0.92). Age at baseline was not significantly related to baseline IMT after adjusting for weight for age z score (separately in cases and controls) (p=0.44), that is any differences according to age in the bivariable analyses could be explained by other variables. There was also no significant residual effect of recruitment site in experienced children (p=0.97). Despite the strong effects of weight for age z score, the residual effect of HIV case vs uninfected control remained, with IMT 0.033mm higher in HIV-infected cases after adjustment (p=0.03).

5.6 Longitudinal changes in IMT

5.6.1 Impact of missing data

Table 5-8 summarises available data over time. As at baseline, the main reason for missing scans was younger age and therefore completeness is higher in older children (group 2 and group 1 at later time points). UTH also experienced more technical difficulties in successfully scanning children, especially at earlier time points. Consequently the regression models used for longitudinal analysis adjusted for age and site.

Table 5-8. Summary of scans performed by week of follow up.

Group 1	Week 0	Week 48	Week 96
UTH			
Number in trial	89	70	67
Number with IMT (%)	39 (44)	44 (63)	50 (75)
Number with missing IMT (%)	50 (56)	26 (37)	17 (25)
Died / LTFU		19	3
JCRC			
Number in trial	119	115	112
Number with IMT (%)	106 (89)	103 (90)	93 (83)
Number with missing IMT (%)	13 (11)	12 (10)	19 (17)
Died / LTFU		4	3

Group 2			
UTH			
Number in trial	52	52	52
Number with IMT (%)	51 (98)	51 (98)	50 (96)
Number with missing IMT (%)	1 (2)	1 (2)	2 (4)
JCRC			
Number in trial	22	22	22
Number with IMT (%)	21 (96)	22 (100)	19 (86)
Number with missing IMT (%)	1 (5)	0	3 (14)

IMT, intimal medial thickness; LTFU, lost to follow up.

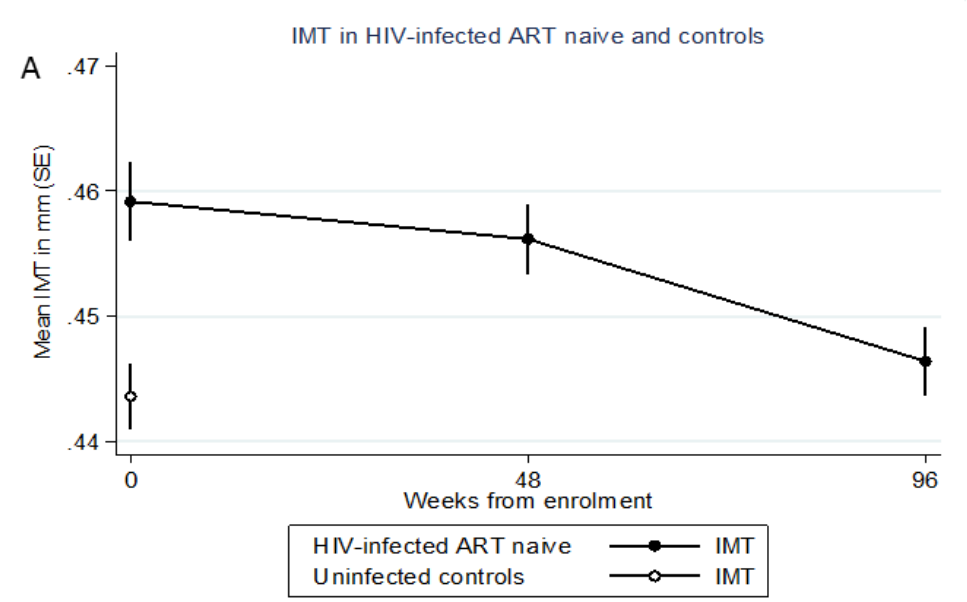
5.6.2 Longitudinal changes in IMT

Over 96 weeks from baseline significant improvements in IMT were demonstrated in both ART naïve and ART experienced children. As summarized in Table 5-9 and illustrated in Figure 5-9 a significant decrease of (-)0.02mm (sd 0.04) ($p<0.001$) and (-)0.02mm (sd 0.06) ($p=0.01$) in the IMT from baseline was seen in ART naïve / experienced children respectively.

Table 5-9. Summary of longitudinal changes in IMT by group.

Group 1 – ART naive		Week 0	Week 48	Week 96
Total number IMT performed at time point		145	147	143
Mean IMT (SD) in mm		0.46 (0.04)	0.46 (0.04)	0.45 (0.03)
Mean change between week 0 and 48	n = 123	-0.004 (0.03), p = 0.25		
Mean change between week 0 and 96	n = 116	-0.02 (0.04), **p = 0.0001**		

Group 2 – ART experienced		Week 0	Week 48	Week 96
Total number IMT performed at time point		72	73	69
Mean IMT (SD) in mm		0.46 (0.05)	0.45 (0.05)	0.44 (0.04)
Mean change between week 0 and 48	n = 72	-0.01 (0.05), p = 0.07		
Mean change between week 0 and 96	n = 68	-0.02 (0.06), **p = 0.01**		



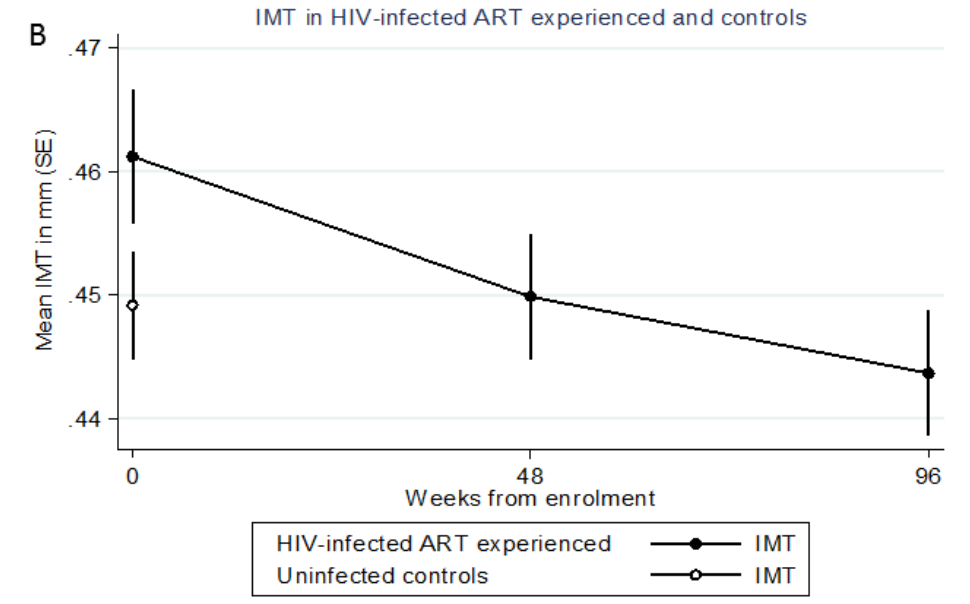


Figure 5-9. Change in IMT over 96 weeks

A) HIV infected, ART naïve (initiating ART) and controls and B) HIV infected, ART experienced and controls.

5.7 Impact of ART regimen on changes in IMT

Adjusted regression analysis was used to investigate any effects of randomized NRTI on change in IMT over 48 and 96 weeks. All models included baseline IMT as a factor to adjust for regression to the mean effects. As overall changes in IMT were similar in naïve and experienced children (Figure 5-8), I pooled both groups to gain the greatest power to detect any differences between randomized NRTI. I then checked that this pooled analysis had not masked differential effects of randomized NRTI according to whether the child was naïve or experienced by testing for significant interactions between randomized NRTI and naïve/experienced strata. There was no evidence of any interaction between randomized NRTI and naïve/experienced strata at either week 48 or 96 ($p>0.05$), and so results from main effects models are presented.

There was no evidence of a difference between randomized groups in change in IMT from baseline to week 48 adjusting for site, age and baseline IMT (global test across the three arms ($p=0.27$)). In particular there was no evidence of a difference between ABC and d4T ($p=0.98$) or between ABC and ZDV ($p=0.16$).

Similarly there was no evidence of a difference between randomized groups in change in IMT from baseline to week 96 adjusting for site, age and baseline IMT (global test across the three arms $p=0.77$). In particular there was no evidence of a difference between ABC and d4T ($p=0.47$) or between ABC and ZDV ($p=0.76$) - Figure 5-10.

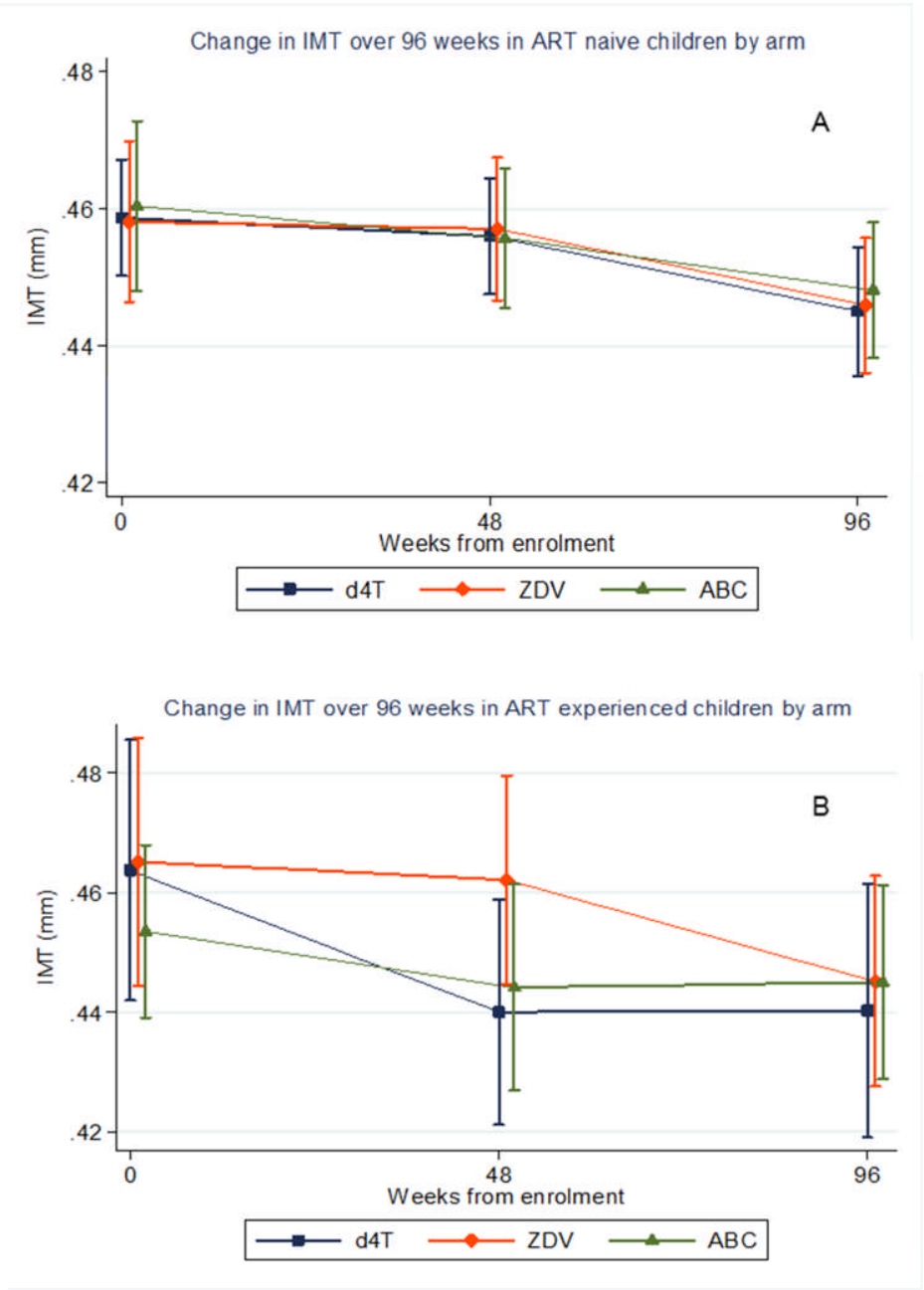


Figure 5-10. Changes in IMT over 96 weeks by NRTI randomisation arm.

A) ART naïve children and B) ART experienced children over the 96 weeks after randomisation to either d4T, ZDV or ABC. No significant differences by NRTI randomisation arm were demonstrated. Mean and SD shown.

5.8 Impact of baseline variables on IMT at week 0, 48 and 96.

A univariable linear mixed effects model was used to model the absolute IMT values at weeks 0, 48 and 96 jointly, including one single random effect per child (reflecting an individual child's value versus the population mean), in order to estimate a common effect of other baseline factors on absolute IMT at the three time points. As listed in Table 5-10 48 weeks of follow up ($p=0.05$), 96 weeks of follow up ($p<0.001$) and baseline urea ($p<0.001$) were significantly associated with IMT at the measured time points. As only time point and baseline urea had $p<0.05$ in univariable models, one final multivariable model was fitted including these two variables ($p<0.05$) and other variables known to provide important information for the study populations (age, sex, site and HIV naïve/experienced). The highly significant effect of urea and no other variable is surprising. When I added urea into the previous multivariable model described in section 0, fitted only to the naïve HIV-infected children, urea did not have an independent effect, suggesting that its highly significant effect in this model may be a chance spurious finding, given the large number of statistical tests performed.

Table 5-10. Association between the clinical characteristics of the study population on IMT at week 0, 48 and 96 using a univariable linear mixed effects model.

Variable	Estimate	95% CI	p value
Patient details			
Age (years)	0.00	(0.00 : 0.00)	0.31
Sex	-0.01	(-0.01 : 0.00)	0.06
Site	0.00	(-0.01 : 0.01)	0.55
Week of follow up - 48	-0.01	(-0.01 : 0.00)	**0.05**
Week of follow up - 96	-0.02	(-0.02 : -0.01)	**<0.001**
Baseline Weight-for-age Z	0.00	(0.00 : 0.00)	0.23
Baseline Height-for-age Z	0.00	(0.00 : 0.00)	0.21
Baseline BMI-for-age Z	0.00	(0.00 : 0.00)	0.71
Baseline mean sBP (per 5mmHg)	0.00	(0.00 : 0.00)	0.27
Baseline mean dBP (per 5mmHg)	0.00	(0.00 : 0.00)	0.32
Baseline mean HR	0.00	(0.00 : 0.00)	0.36
HIV related			
ART (naïve v experienced)	0.00	(-0.01 : 0.00)	0.47
NNRTI - NVP v EFV	0.00	(-0.01 : 0.01)	0.73
ARM – ZDV v d4T	0.00	(-0.01 : 0.01)	0.44
ARM – ABCv d4T	0.00	(-0.01 : 0.01)	0.67
Baseline CD4	0.00	(0.00 : 0.00)	0.40
Baseline CD4%	0.00	(0.00 : 0.00)	0.19
Baseline CD4 z score	0.00	(0.00 : 0.00)	0.62
Current WHO 2 v 1	0.00	(-0.01 : 0.01)	0.76
Current WHO 3 v 1	0.00	(-0.01 : 0.01)	0.58
Current WHO 4 v 1	-0.01	(-0.02 : 0.01)	0.39
Previous WHO grade 3/4	0.00	(-0.01 : 0.01)	0.77
Baseline VL	0.00	(0.00 : 0.00)	0.80
Age started ART (years)	0.00	(0.00 : 0.00)	0.47
Duration on ART (years)	0.00	(0.00 : 0.00)	0.44
Laboratory results			
Baseline total cholesterol	0.00	(-0.01 : 0.00)	0.47
Baseline triglycerides	0.00	(0.00 : 0.01)	0.64
Baseline LDL	0.00	(-0.01 : 0.00)	0.41
Baseline HDL	0.00	(-0.01 : 0.01)	0.64
Baseline urea	-0.01	(-0.01 : 0.00)	**<0.001**
Baseline creatinine	0.00	(0.00 : 0.00)	0.47
Baseline MCV	0.00	(0.00 : 0.00)	0.11
Baseline haemaglobin	0.00	(0.00 : 0.00)	0.26
Baseline platelets	0.00	(0.00 : 0.00)	0.21

BMI, body mass index; sBP, systolic blood pressure; dBP, diastolic blood pressure; HR, heart rate; NNRTI, non-nucleoside reverse transcriptase; NVP, nevirapine; EFV, efavirenz; ZDV, zidovudine; d4T, stavudine; ABC, abacavir; WHO, World Health Organisation; VL, viral load; LDL, low density lipoprotein HDL , high density lipoprotein; MCV, mean corpuscular volume.

Table 5-11. Multivariable model for absolute IMT at week 0, 48 and 96 weeks.

Variable	Estimate	95% CI	p value
Patient details			
Age (years) at baseline	0.00	(0.00 : 0.00)	0.14
Sex - female v male	-0.01	(-0.01 : 0.00)	0.07
Site - JCRC v UTH	0.01	(0.00 : 0.02)	**0.05**
Week of follow up - 48 v 0	-0.01	(-0.01 : 0.00)	**0.05**
Week of follow up - 96 v 0	-0.02	(-0.02 : -0.01)	**<0.001**
HIV related			
ART experienced v naïve	0.00	(-0.01 : 0.01)	0.80
Laboratory results			
Baseline urea	-0.01	(-0.01 : 0.00)	**<0.001**

In the multivariable analysis (Table 5-11), after adjusting for age, sex, site and HIV naïve/experienced), IMT was significantly lower after 48 / 96 weeks by -0.01 / -0.02mm, 95% CI (-0.01 : 0.00 / -0.02 : -0.01), (p=0.04 / <0.001). Children at UTH had an IMT -0.01mm lower than children at JCRC, 95% CI (-0.02: -0.00), (p=0.05). For every umol/l higher baseline urea IMT was 0.01mm lower 95% CI (-0.01 : 0.00), (p<0.001). There was no evidence of an interaction between HIV-infection and age or urea (p>0.05).

5.9 Impact of baseline variables on the change in IMT from baseline to week 96

The previous analysis considered all values of IMT together in one model. However, the particular interest in this trial was the change from baseline to the primary endpoint time of week 96. I therefore considered separate models just for this change (one observation per child), following the previous modelling strategy used to identify predictors of baseline IMT. Some additional factors were included in these models that had not been measured in controls (e.g. biochemistry) and so were not considered in the earlier models. Specifically, a bivariable main effects and interaction model (allowing a different effect of each factor depending on whether the child was ART naïve or experienced at baseline), also including baseline IMT to adjust for regression to the mean, were used to screen the effects of other baseline variables on the change in IMT over 96 weeks -Table 5-12. Any variable or interaction which had $p<0.2$ in the main effects or interaction model was included in a multivariable model from which a final multivariable model was identified using backward elimination (exit $p=0.05$). ART naïve/experienced status, age, baseline IMT and site of recruitment were included in all models regardless of significance as the key exposure of interest (ART naïve v experienced) or because they were factors a priori considered to have potential influence. After a model had been identified by backwards elimination, each rejected main effect and interaction term was checked for additional prognostic importance given this model, and any factors with $p<0.05$ retained.

Table 5-12. Results of a bivariate analysis, using a main effects and interaction model to screen for effects of baseline variables on the change in IMT from baseline to week 96.

	Relationship to change in IMT over 96 week		
	p value for main effect adjusted for group	p value for interaction by ART naïve / experienced	
Clinical variables at baseline			
Age in years	**0.13**	0.29	
Sex	0.77	**0.15**	
Site	0.96	1.00	
Weight-for-age Z	0.84	0.41	
Height-for-age Z	0.86	0.66	
BMI-for-age Z	0.78	0.34	
Mean sBP	0.31	0.98	
Mean dBP	0.48	0.86	
Mean HR	0.79	0.89	
HIV related variables			
Baseline WHO	0.37	2 v 1 3 v 1 4 v 1	0.57 0.47 **0.05**
Baseline log10 VL	0.96	NA	
Wk 96 VL suppression	0.41	0.75	
Duration on ART at baseline	0.76	NA	
NNRTI - NVP v EFV	0.47	0.45	
ARM – ZDV v d4T ARM – ABCv d4T	0.38	2 v 1 3 v 1	0.69 0.75
Immunological variables at baseline			
CD4	0.29	0.84	
CD4%	0.43	0.51	
CD4 z score	0.35	0.82	
Lipids at baseline			
Total cholesterol	0.77	**0.11**	
Triglycerides	0.68	**0.01**	
LDL	0.87	0.22	
HDL	0.50	**0.18**	
Laboratory markers at baseline			
Urea	**0.06**	0.48	
Creatinine	0.87	0.98	
MCV	0.26	0.62	
Haemaglobin	0.57	0.51	
Platelets	0.45	**0.16**	

Main effects model included HIV status and factor of interest. Interaction model includes main effects model and interaction between HIV status and factor of interest. Absolute CD4 count and CD4 % were truncated at 99th centile. BMI, body mass index; dBP, diastolic blood pressure; HDL, high density lipoprotein; HR, heart rate; LDL, low-density lipoprotein; NA, not applicable; sBP, systolic blood pressure; Wk, week; VL, viral load.

The final multivariable model after backward elimination (Table 5-13) looked at mean change over 96 weeks in IMT compared to the reference category (reference category age 3 years, triglycerides 1mmol/l, ART-naïve at baseline). For each 1 year older at baseline, the change in IMT from baseline to week 96 was (+)0.003mm larger (p=0.04) (i.e. any decline was smaller, or even an increase). Every 1 mmol/l higher baseline urea was associated with an (-)0.07mm greater decline in IMT from baseline to week 96 (p=0.01).

WHO stage 4 HIV-disease at baseline was significantly associated with a greater improvement (-)0.048 mm in IMT over 96 week in children who were ART experienced at baseline (p=0.01). Despite the strong effect of total triglycerides, a residual effect of being an ART naive vs ART experienced at baseline remained, the change in IMT over 96 weeks was an increase of 0.009mm for each 1mmol/l increase in triglycerides in ART naïve children (p=0.05) and a decrease of (-)0.021mm for each 1 mmol/l increase in triglycerides in ART experienced children (p=0.003).

Table 5-13. Final multivariable models for change in IMT from baseline to week 96.

Variable		Estimate of impact on change in IMT (mm)	95% CI	p value
Mean at reference category		0.319	(0.245 : 0.394)	**<0.001**
ART at baseline - experienced v naïve		0.008	(-0.013 : 0.029)	0.44
Age at baseline (per year older)		0.003	(0.000 : 0.006)	**0.04**
Site UTH v JCRC		-0.002	(-0.018 : 0.013)	0.77
Baseline urea (per 1 mmol/l increase)		-0.007	(-0.012 : -0.001)	**0.01**
Baseline WHO stage in children ART naïve at baseline				
	2 v 1	0.004	(-0.015 : 0.022)	0.68
	3 v 1	0.000	(-0.017 : 0.018)	0.96
	4 v 1	0.023	(-0.027 : 0.073)	0.37
Baseline WHO stage in children ARTexperienced at baseline				
	2 v 1	-0.027	(-0.060 : 0.007)	0.12
	3 v 1	-0.015	(-0.043 : 0.013)	0.29
	4 v 1	-0.048	(-0.082 : -0.013)	**0.01**
Baseline triglycerides in children ART naïve at baseline (per 1 mmol/l increase)		0.009	(0.000 : 0.018)	**0.05**
Baseline triglycerides in children ART experienced at baseline (per 1 mmol/l increase)		-0.021	(-0.035 : -0.007)	**0.003**

Models were adjusted for baseline IMT. Reference category; age = 3 years, triglycerides = 1 mmol/l. ART, anti-retroviral therapy; IMT, intimal medial thickness; JCRC, Joint Clinical Research Centre; UTH, university teaching hopsital; WHO, World Health Organisation.

5.10 Pulse Wave Velocity (PWV)

5.10.1 Missing baseline PWV by site and group

Technical difficulties and uncooperative children meant some recruits could not be scanned; this tended to be the younger children, Table 5-14. Comparing the median age of children in groups 1 and 2 with baseline PWV scans with their control group respectively showed that at UTH the children with PWV results in group 1 were significantly older (median age 6.3 years) than the control group (median age 4.2 years) (p=0.002). This can be explained as the controls were recruited later in the study when the clinical team was more confident in scanning smaller children. No differences were seen between Group 2 and controls at UTH (p=0.59) nor at JCRC between both group 1/2 and controls (p=0.12/0.82). Combining all children recruited from both sites to group 1/2 no difference was seen between groups and controls (p=0.59/0.55). Thus whilst comparisons of HIV infected versus controls cannot be confounded by age as a whole, further analyses that adjust for site will also be adjusted for age.

Table 5-14. Summary of missing baseline PWV recordings by group and site.

	HIV+ ART naïve (group 1)	HIV- controls matched to group 1	HIV+ ART experienced (group 2)	HIV- controls matched to group 2
UTH				
Total recruited	89	89	52	52
Missing PWV (% total)	51 (57)	29 (33)	1 (2)	0
Age	2.9 (1.8 : 6.1)	3.4 (2.1 : 5.6)	7.0 (5.8 : 8.2)	6.9 (5.6 : 8.3)
Age with a baseline PWV	6.3 (4.5 : 9.6)	4.2 (3.0 : 6.4)	7.0 (5.8 : 8.5)	6.9 (5.6 : 8.3)
Age with missing baseline PWV	1.8 (1.2 : 2.6)	1.5 (0.9 : 2.7)	5.5	
JCRC				
Total recruited	119	120	22	23
Missing PWV (% total)	16 (13)	25 (21)	0	0
Age	2.8 (1.6 : 4.0)	2.9 (2.1 : 4.1)	6.5 (5.9 : 9.9)	6.4 (5.5 : 9.1)
Age with a baseline PWV	3.2 (1.8 : 4.3)	3.3 (2.4 : 4.4)	6.5 (5.9 : 9.9)	6.4 (5.5 : 9.1)
Age with missing baseline PWV	1.5 (1.1 : 2.2)	1.8 (1.3 : 2.6)		

5.11 Baseline Pulse Wave Velocity

Two results from group 1 were excluded; these were both <3m/sec, performed in young children on the same day and considered not to be physiologically possible. One extremely high result (PWV – 9.9m/sec) was also obtained in group 1 at week six; this could be biologically possible; the patient at that visit also had a raised CRP and was febrile so potentially this was a true reading but reflecting an intercurrent infection. However this could also heavily influence results so this result (and others >99th centile) were truncated and replaced with the 99th centile value in subsequent analyses. To determine the impact of scans performed after week 0 firstly the results were plotted - Figure 5-11. Regression analysis confirmed there was no trend in baseline PWV by time after randomization in either group 1 or group 2 (p=0.18/0.97) so all results were included in the further analyses.

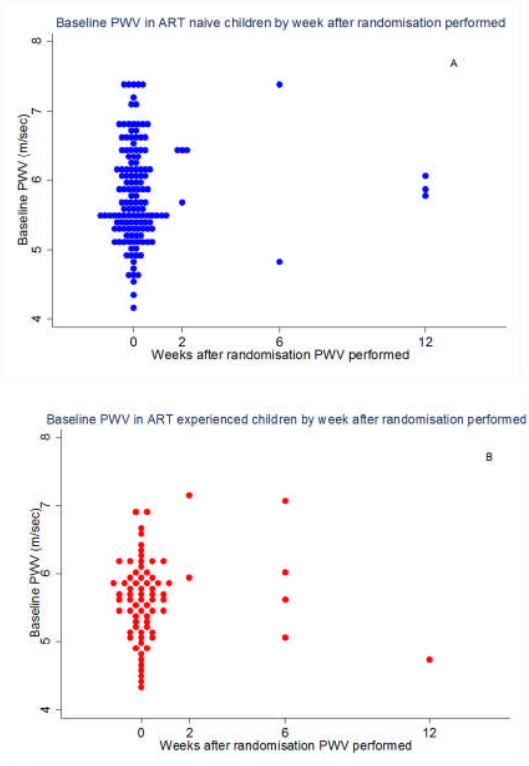


Figure 5-11. Baseline PWV results

A) ART naïve and B) ART experienced children by week after randomisation performed.

As summarized in Table 5-15 and illustrated in Figure 5-12 baseline PWV was significantly higher in group 1 versus controls (p=0.05). No significant difference was seen between the smaller numbers in group 2 and controls (p=0.57). Results after excluding nine and seven scans performed at weeks 2 -12 after enrollment in groups 1 and 2 respectively showed the same trend towards a significant difference between group 1 and controls (p=0.09) and no difference between group 2 and controls (p=0.38) As there was no significant time trend in PWV with increasing time post-enrolment, and because power to detect differences between cases and controls will be reduced restricting to the fewer children with scans at week 0, I have included all scans up to 12 weeks in further analyses.

Table 5-15. Baseline PWV results.

	HIV+ ART naïve group 1	**HIV- controls matched to Group 1**	**HIV+ ART experienced group 2**	**HIV- controls matched to Group 2**
Total number in group	208	209	74	75
Overall mean (SD)		5.723 (0.73)		
Median (IQR)		5.673 (5.188 – 6.20)		
Number PWV scans performed	141	155	73	75
Mean in m/sec (SD)	5.85 (0.80)	5.67 (0.74)	5.63 (0.61)	5.69 (0.68)
Range	4.12 : 9.89	4.13 : 7.26	4.34 : 7.15	4.07 : 7.33
Number performed after week 0 (% total)	9		7	
	Wk 2 4 (3%)		Wk 2 2 (3%)	
	Wk 6 2 (1%)		Wk 6 4 (5%)	
	Wk 12 3 (2%)		Wk 12 1 (1%)	
Missing (% total)	67 (32%)	54 (26%)	1 (1%)	
Too young (% missing)	57 (85%)	46 (85%)		
Not done	7(10%)	5 (9%)	1 (100%)	
Unanalysable				
Missing*	3 (5%)	3 (6%)		

*IQR, interquartile range; PWV, pulse wave velocity; SD, standard deviation. * missing recordings – were performed but accidently deleted prior to data transfer*

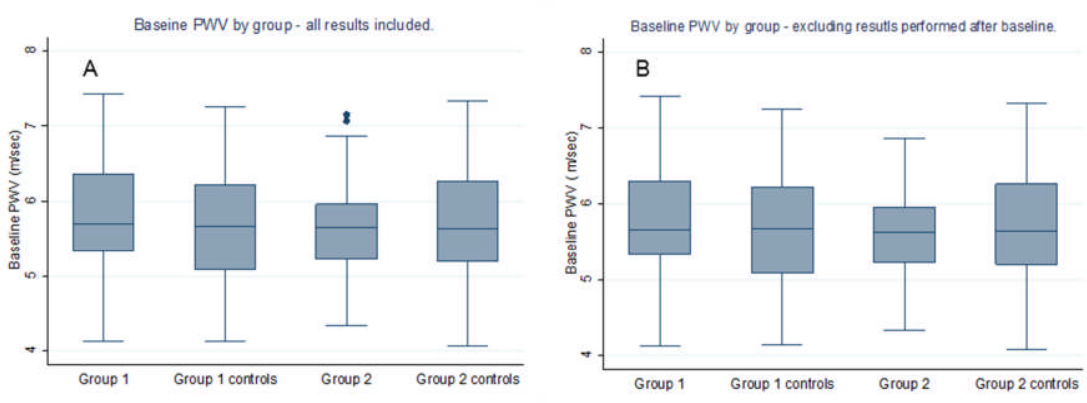


Figure 5-12. Boxplot of baseline PWV between the groups.

Median and IQR given. A) results when all baseline PWV results are included, B) results when PWV recordings performed after week 0 are excluded.

5.12 Impact of variables on baseline PWV

5.12.1 Age

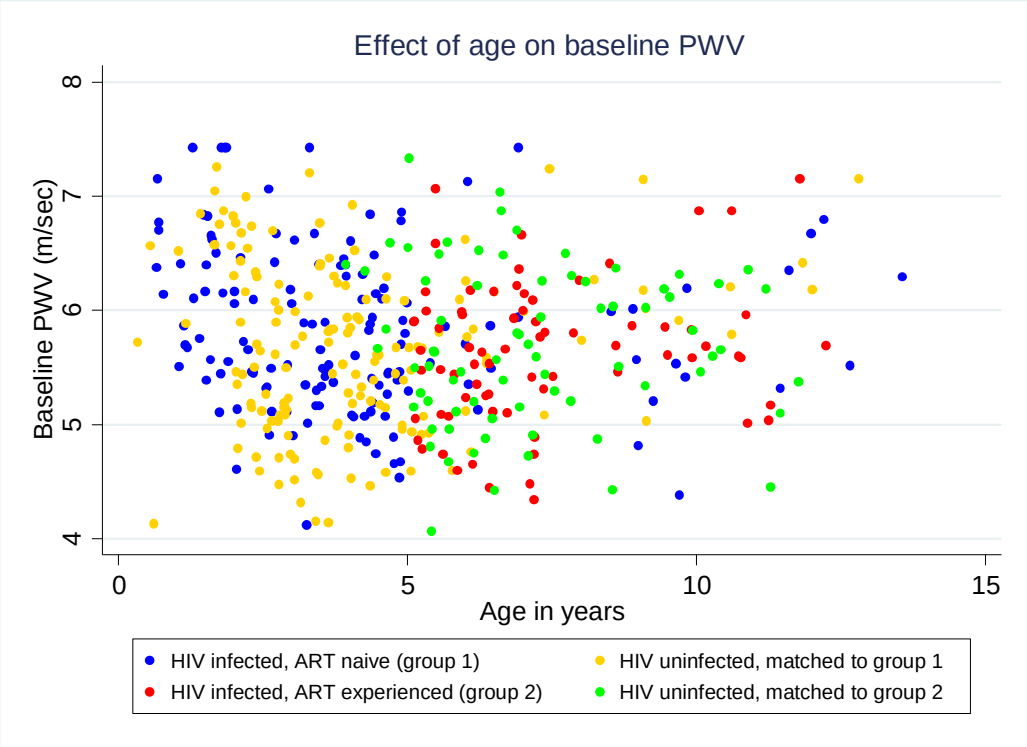


Figure 5-13. Effect of age on baseline PWV by group.

Comparing group 1 (ART naïve) and their control group in a full interaction model, allowing the relationship between baseline PWV and age to vary by HIV infection, estimates were that in the ART-naïve children baseline PWV was (-)0.06 lower for every year older (95% CI -0.08 : +0.13) (p=0.08), and in the controls baseline PWV was 0.03 higher for every year older (95% CI -0.03 : +0.08) (p=0.31). There was no significant evidence that the impact of age at baseline varied by HIV status (interaction p=0.08). Fitting the same relationship between age and baseline PWV in both groups there was still no evidence of a relationship between baseline PWV and age overall –

baseline PWV was (-)0.007 lower per year older (95% CI -0.04 : +0.03), (p=0.67). After adjusting for age, there was a trend towards PWV being (-)0.16m/second lower in controls (95% CI -0.32 : +0.01) (p=0.06).

Comparing group 2 (ART experienced) and their control group in a full interaction model, allowing the relationship between baseline PWV and age to vary by HIV infection, estimates were that in the ART-experienced children baseline PWV was 0.06 higher for every year older (95% CI -0.02 : +0.14) (p=0.13), and in the controls baseline PWV was (-)0.01 lower for every year older (95% CI -0.08 : +0.07) (p=0.83). There was no evidence that the impact of age at baseline varied by HIV status (interaction p=0.22). Fitting the same relationship between age and baseline PWV in both groups there was still no evidence of a relationship between baseline PWV and age overall – baseline PWV was 0.03 higher per year older (95% CI -0.03 : +0.08), (p=0.37). No significant association between baseline PWV and HIV status was seen after adjusting for age (PWV 0.07m/second higher in controls (95% CI -0.15: 0.28) (p=0.54)).

In the ART experienced group, duration of ART prior to baseline ranged from 2 – 7.1 years (median 3.9 years, IQR (2.9 : 4.3)). As illustrated in Figure 5-14 the distribution of age at baseline and duration of ART at baseline was varied (Spearman rho=0.02, p=0.86) and therefore it was possible to estimate an effect of duration of ART on PWV independently of any effect of age at enrolment. Regression analysis showed that for each year on ART, after adjusting for age, PWV was (-)0.03m/sec lower but this was not significant (95%CI (-0.16 : +0.11), p=0.71). There was also no effect in a univariable analysis not adjusted for age (p=0.70).

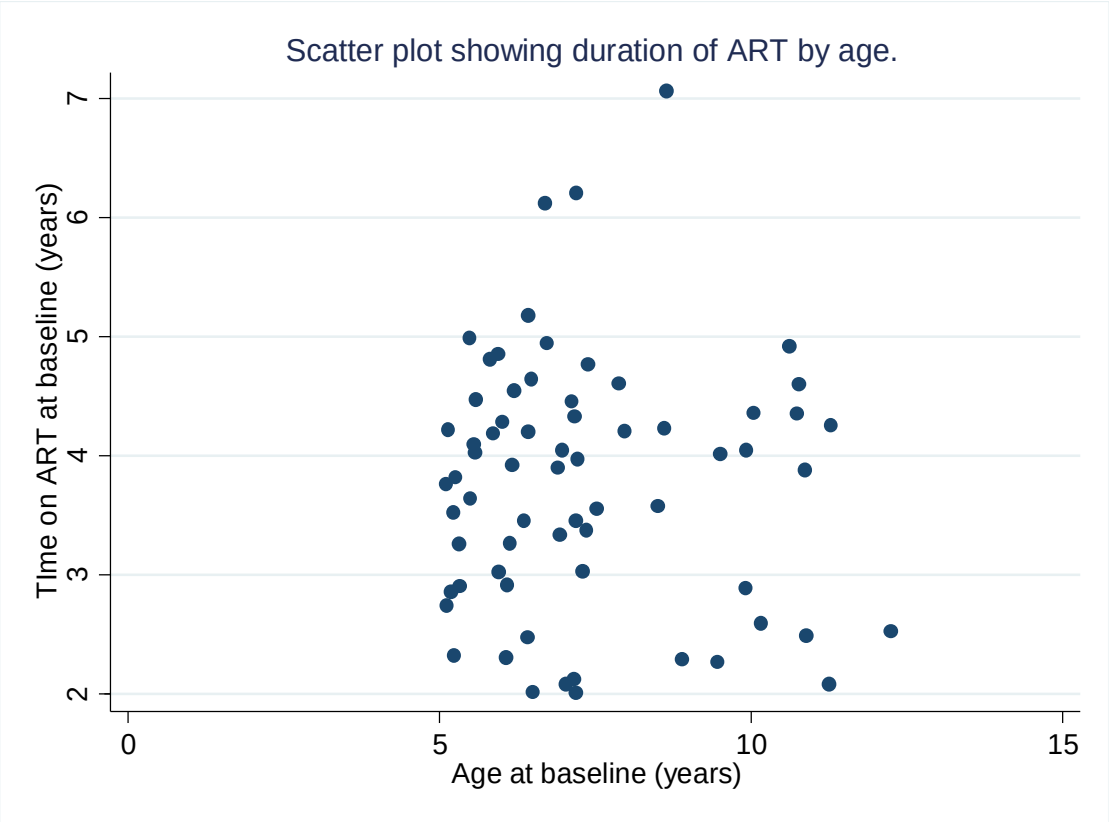


Figure 5-14. The relationship between duration on ART and age at baseline.

A wide variation exists with no significant relationship (Spearman co-efficient = 0.02, p=0.86)

5.13 Effect of age on PWV in HIV uninfected controls

As for IMT there are no published normal ranges of PWV in young children. To attempt to calculate a normal range PWV was plotted against age in all 230 HIV uninfected controls - Figure 5-15. The point estimate suggests that PWV increases by 0.01m/sec for each additional year of age but there is no formal evidence that this is not compatible with chance (95% CI -0.02 : 0.05), (p=0.45).

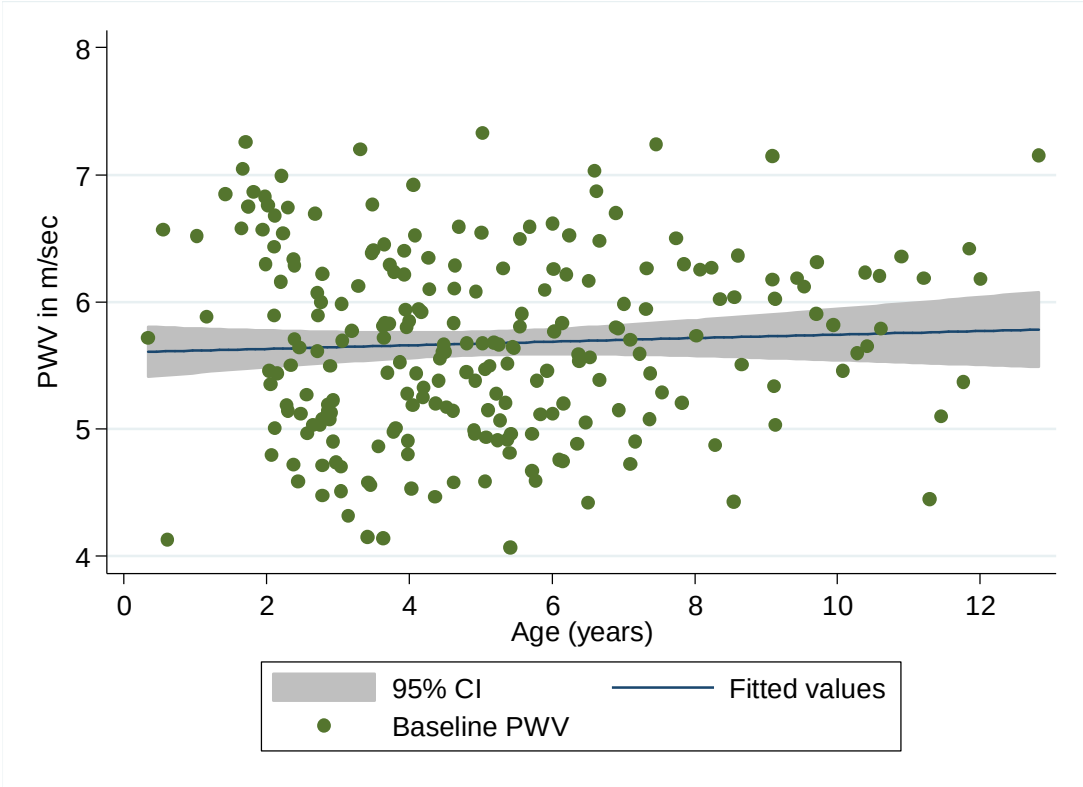


Figure 5-15. Effect of age on PWV in HIV uninfected children,

No significant impact of age on PWV was demonstrated (95% CI -0.02 : 0.05), (p=0.45).

5.13.1 Impact of baseline CD4 count on baseline PWV

No significant associations were demonstrated between absolute CD4 count and PWV in either ART-naïve and controls or ART-experienced and controls (p=0.37/0.13 respectively). A significant inverse association was seen between CD4 percentage and PWV in both ART-naïve and controls and ART-experienced and controls (r = -0.21 / -0.20), (p<0.001/0.02) (not adjusting for other factors), Figure 5-16.

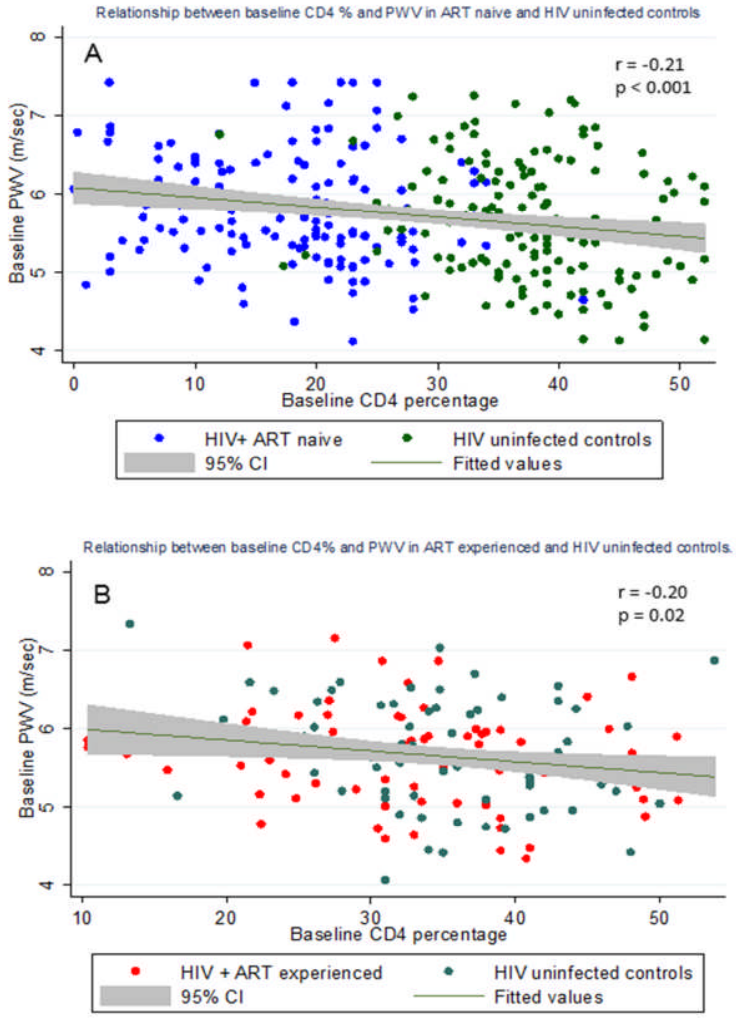


Figure 5-16. The relationship between baseline CD4 percentage and PWV

A) ART naïve children and their controls and B) ART experienced children and their controls.

5.13.2 Impact of other variables on baseline PWV.

A bivariable main effects and interaction model were used to screen the effects of variables on baseline PWV - Table 5-16. Any variable or interaction which had $p<0.2$ in the main effects or interaction model was included in the multivariable analysis, using backwards elimination to select a final model (exit $p=0.05$). Case/control status, age and site of recruitment were included in all models regardless of significance as they were the key exposure of interest (case vs control) or factors a priori considered to have potential influence. After a model had been identified by backwards elimination, each rejected main effect and interaction term was checked for additional prognostic importance given this model, and any factors with $p<0.05$ retained.

Table 5-16. Results of a bivariable analysis, using a main effects and interaction model to screen for the effects of variables on baseline PWV.

	ART naïve versus controls		ART experienced versus controls	
	p value for main effect adjusted for group	p value for interaction by HIV status	p value for main effect adjusted for group	p value for interaction by HIV status
Clinical variable				
Age in years	0.67	**0.08**	0.37	0.22
Sex	0.36	0.53	0.70	0.50
Site	**0.01**	**0.02**	**<0.001**	0.31
Weight-for-age Z	**<0.001**	**0.01**	0.77	0.24
Height-for-age Z	**0.002**	**0.01**	0.27	0.51
BMI-for-age Z	**0.02**	**0.03**	0.29	0.35
Mean sBP	**0.01**	0.98	**<0.001**	0.58
Mean dBP	0.38	0.52	**<0.001**	0.60
Mean HR	**0.12**	**<0.001**	0.32	0.92
HIV related variables				
Current WHO	0.45	NA	**0.05**	NA
Baseline log10 VL	**0.16**	NA	NA	NA
Duration on ART	**NA**	NA	0.70	NA
Immunological variable				
CD4	0.92	0.42	0.26	0.21
CD4%	**0.001**	0.31	**0.02**	0.40
CD4 z score	0.26	0.62	0.56	0.24
Lipids				
Total cholesterol	0.63	**0.18**	0.27	0.73
Triglycerides	**0.004**	0.77	0.54	0.98
LDL	0.43	0.35	0.50	0.75
HDL	0.88	**0.06**	**0.09**	0.96

Controls were classed as WHO stage 1, viral load = 100 and for those matched to group 2 (ART experienced) time on ART as 2 years. Main effects model included HIV status and factor of interest. Interaction model includes main effects model and interaction between HIV status and factor of interest. Absolute CD4 count and CD4 % were truncated at 99th centile. BMI, body mass index; dBP, diastolic blood pressure; HDL, high density lipoprotein; HR, heart rate; LDL, low-density lipoprotein; NA, not applicable; sBP, systolic blood pressure; VL, viral load.

HIV infected ART naïve versus control children

The final multivariate model in ART naïve (group 1) v controls after backward elimination (Table 5-17) included baseline CD4 percentage (PWV -0.02m/sec lower for every 1% higher CD4 percentage (p=0.002), baseline systolic blood pressure (PWV -0.02m/sec lower for every 1mmHg higher, (p<0.001) and baseline triglycerides (PWV 0.16m/sec higher for every mmol/L higher triglycerides (p=0.01). There was a trend towards higher PWV in HIV-infected children after adjusting for age, site, CD4 percentage, systolic blood pressure and triglycerides (p=0.06). Despite strong effects of baseline CD4 percentage, triglycerides and systolic blood pressure there were still residual effects of site; the PWV of children recruited at UTH was 0.28m/sec higher than children recruited at JCRC (p=0.01) and age; PWV 0.14m/sec higher per year older (p<0.001) in HIV-infected children. In HIV uninfected children no significant effect of age was demonstrated (p=0.18; interaction p=0.08). There was no evidence of collinearity between systolic blood pressure and the other variables included in the model.

Table 5-17. Final multivariable models for baseline PWV in HIV infected ART naïve children and controls.

	Estimate of impact on PWV (m/sec)	95% CI	p value
Naïve & controls			
Mean at reference category	7.921	(7.014 : 8.828)	<0.001
HIV-infected case vs uninfected control	0.364	(-0.012 : 0.740)	0.06
Site (UTH vs JCRC)	0.280	(0.059 : 0.501)	**0.01**
CD4 percentage (per 1% increase)	-0.018	(-0.029 : -0.007)	**0.002**
Systolic BP (per 1mmHg higher)	-0.018	(-0.026 : -0.009)	**<0.001**
Triglycerides (per 1mmol/l higher)	0.155	(0.043 : 0.266)	**0.01**
Age in HIV infected (per 1 year older)	0.138	(0.065 : 0.211)	**<0.001**
Age in controls (per year older)	0.039	(-0.019 : 0.097)	0.18

Reference category = age 0. BP. Blood pressure; IMT, Intimal Medial Thickness; JCRC, Joint Clinical Research Centre, Kampala; UTH, University Teaching Hospital, Lusaka;

HIV infected ART experienced versus control children

The final multivariate model in ART experienced (group 2) v controls after backward elimination (Table 5-18) found a significant association of diastolic blood pressure with baseline PWV; PWV was 0.03 m/sec higher for every 1mmHg higher diastolic blood pressure ($p<0.001$). Despite the strong effect of diastolic blood pressure there was still a residual effect of site, with children at UTH having a PWV that was 0.54m/sec higher than at JCRC (95% CI 0.34 ; 0.75), $p<0.001$). After adjusting for age, site and diastolic blood pressure no effect of being HIV infected vs control remained ($p=0.29$). There was no effect of CD4 percentage on baseline PWV after adjusting for these other factors.

Table 5-18. Final multivariable models for baseline PWV in HIV infected, ART experienced children and controls.

	Estimate of impact on PWV (m/sec)	95% CI	p value
Experienced & controls			
Mean at reference category	3.578	(2.712 : 4.444)	<0.001
HIV-infected case vs uninfected control	0.097	(-0.085 : 0.280)	0.29
Age (per 1 year older)	0.022	(-0.026 : 0.069)	0.37
Site (UTH vs JCRC)	0.544	(0.340 : 0.747)	**<0.001**
Diastolic BP (per 1mmHg higher)	0.025	(0.011 : 0.039)	**<0.001**

Reference category = age 0. BP. Blood pressure; IMT, Intimal Medial Thickness; JCRC, Joint Clinical Research Centre, Kampala; UTH, University Teaching Hospital, Lusaka;

5.14 Longitudinal changes in PWV

5.14.1 Impact of missing data

Table 5-19 summarises the data available at the different time points. As at baseline, the main reason for missing scans was younger age and therefore completeness was higher in older children (group 2 and group 1 at later time points). UTH also experienced more technical difficulties in successfully scanning children. Consequently age and site of recruitment will be adjusted for in subsequent models.

Table 5-19. Summary of available PWV recordings by week of follow up.

Group 1	**Week 0**	**Week 48**	**Week 96**
UTH			
Number in trial	89	70	67
Number with PWV (%)	38 (43)	45 (64)	49 (73)
Number with missing PWV (%)	51 (57)	25 (36)	18 (27)
Died / LTFU		19	3
JCRC			
Number in trial	119	115	112
Number with PWV (%)	103 (87)	103 (90)	95 (85)
Number with missing PWV (%)	16 (13)	12 (10)	17 (15)
Died / LTFU		4	3

Group 2		**Week 48**	**Week 96**
UTH			
Number in trial	52	52	52
Number with PWV (%)	51 (98)	52	48 (92)
Number with missing PWV (%)	1 (2)		4 (8)
JCRC			
Number in trial	22	22	22
Number with PWV (%)	22	21 (95)	21 (95)
Number with missing PWV (%)		1 (5)	1 (5)

PWV, pulse wave velocity; LTFU, lost to follow up.

5.14.2 Changes in PWV over 96 weeks

As reported in Table 5-20 and illustrated in Figure 5-17, mean PWV significantly decreased by -0.37 (SD 0.82) m/sec ($p<0.001$), in ART naïve children over 96 weeks after starting ART. Conversely in ART experienced children a significant increase of 0.34 (SD 0.62) m/sec in PWV over 96 weeks ($p<0.001$); potentially this could reflect an increase in PWV with increasing age in HIV-infected children on ART, although I did not find evidence to support this in my previous model for baseline PWV.

Table 5-20. Summary of PWV changes over time by group.

Group 1 – ART naive		Week 0	Week 48	Week 96
Number PWV		141	148	144
Mean (SD) m/sec		5.83 (0.73)	5.76 (0.81)	5.48 (0.71)
Mean change between week 0 and 48	n = 121	-0.11 (0.77), p = 0.12		
Mean change between week 0 and 96	n = 112	-0.37 (0.82), **p < 0.001**		

Group 2 – ART experienced		Week 0	Week 48	Week 96
Number PWV		73	73	69
Mean (SD) m/sec		5.63 (0.61)	5.77 (0.65)	5.96 (0.68)
Mean change between week 0 and 48	n = 72	0.13 (0.61), p = 0.08		
Mean change between week 0 and 96	n = 68	0.34 (0.62), **p < 0.001**		

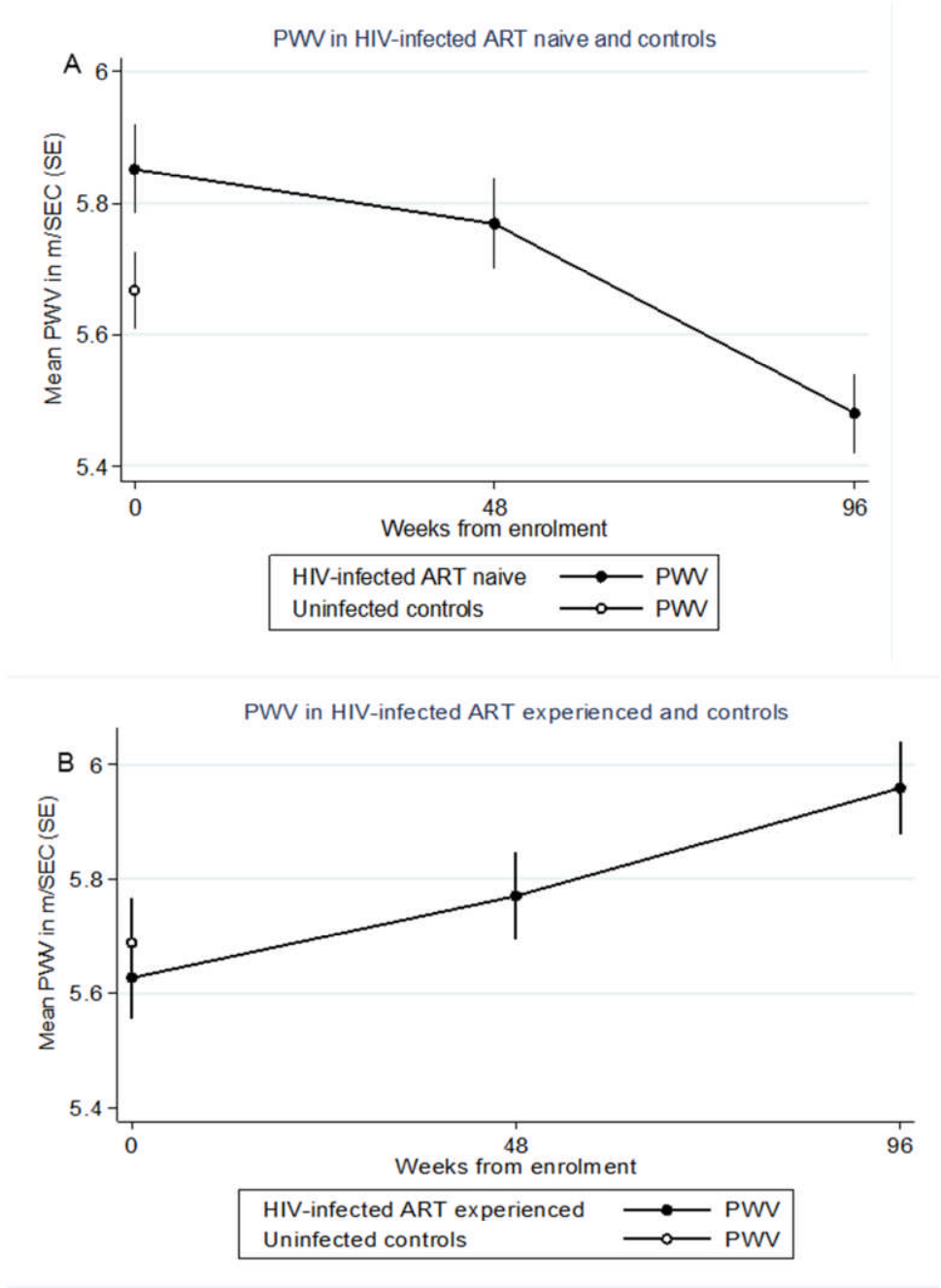


Figure 5-17. Change in PWV over 96 weeks.

A) HIV-infected ART naïve (initiating ART) children and B) HIV infected, ART experienced children.

5.15 Impact of ART regimen on change in PWV over 96 weeks

Adjusted regression analysis was used to look for an effect of randomized NRTI on change in PWV over 48 and 96 weeks. As overall changes in PWV were different in naïve and experienced children (Figure 5-17) a separate model was fitted for each group. Both models included baseline PWV as a factor to adjust for regression to the mean effects.

Within the ART naïve group, after adjusting for site, age and baseline PWV there was no evidence of a difference between randomized groups in change in PWV from baseline to week 48 or week 96 (global test across the three arms p=0.71/0.29). In particular there was no evidence of a difference between ZDV and d4T (p =0.95/0.82), ABC and d4T (p=0.48/0.14) or between ABC and ZDV (p=0.49/0.24).

Within the ART experienced group, after adjusting for site, age and baseline PWV there was no evidence of a significant difference between randomized groups in change in PWV from baseline to week 48 or week 96 (global test across the three arms p=0.09/0.21). Looking in detail by arm a borderline significant results was seen at week 48 comparing ZDV and d4T (p=0.05), with a trend towards a greater increase in PWV from 0 to 48 weeks in the ZDV than the d4T group. However by week 96 no significant difference was seen (p=0.69). Additionally there was no evidence of a difference between ABC and d4T (p=0.07/0.09) or between ABC and ZDV (p=0.93/0.18). Week 48 differences were driven by a mean decrease in the d4T group compared to mean increases in the ZDV and ABC groups. This is illustrated in Figure 5-18.

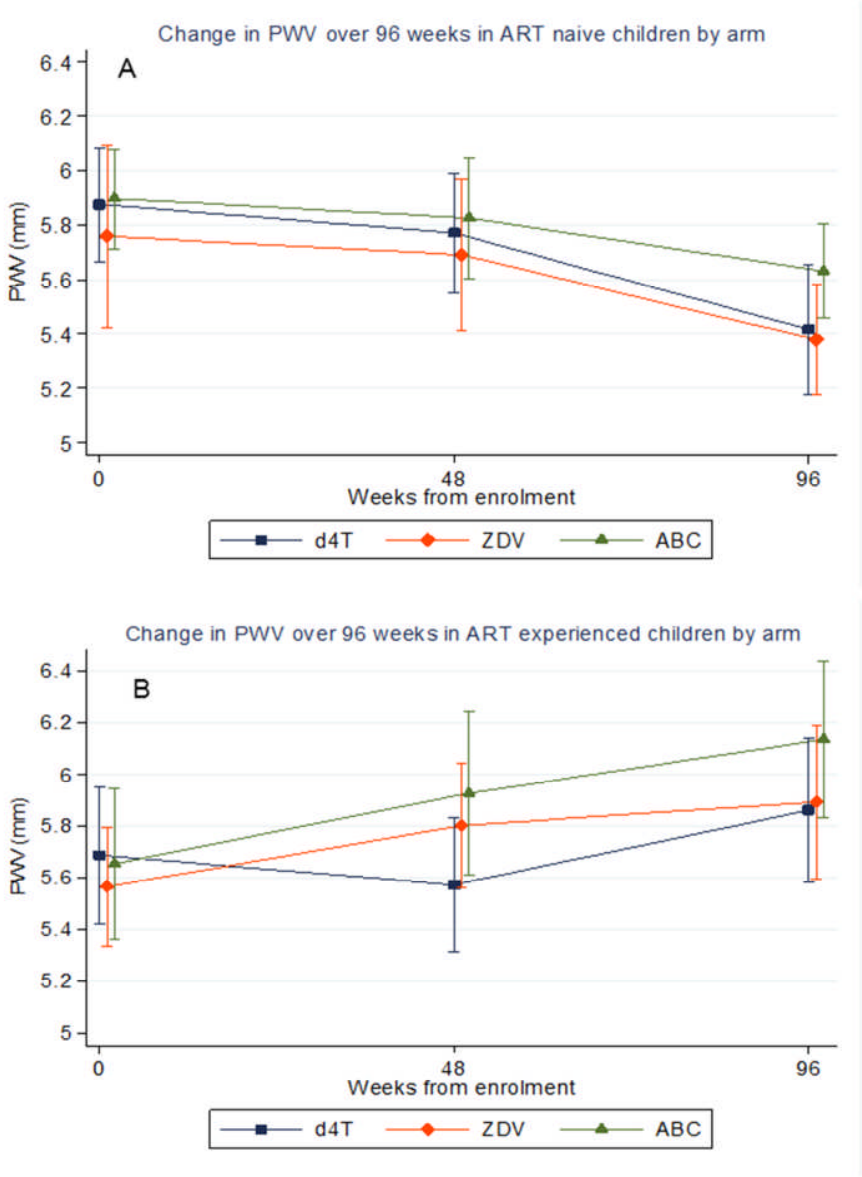


Figure 5-18. Change in PWV over 96 weeks by NRTI randomisation arm.

A) ART naive and B) ART experienced children in the 96 weeks after randomisation to either d4T, ZDV or ABC. Mean and SD given.

5.16 Discussion

5.16.1 IMT results

This is the first report that changes in structural and arterial stiffness are present in very young (median age 2.9 years) HIV infected ART naïve children compared to age matched controls from the same community. These findings compliment the findings of other paediatric studies (summarised in table 2.8) that have found structural changes of the arterial wall in older children and adolescents. Unique to this study were the large number of ART naïve children; the majority with severe immunosuppression (57% of the ART naïve group were classed as WHO stage III/IV) or low CD4 percentage for age (median CD4 percentage 18% (IQR 11 – 23%)).

The ART experienced children were older (median 6.9 years) and virologically suppressed on first line, d4T containing ART, in contrast to other cohorts that have had children taking a heterogeneous selection of ART regimens, including PIs, for variable durations and with different proportions of virological suppression. Having children on a single first line ART combination removed class of ART as a confounder. Two control groups, well matched by age, sex and race – important as these are known to influence IMT and PWV [329-332], added to the strength of the findings.

IMT assesses structural rearrangement of the arterial wall and is a well validated surrogate marker of subclinical atherosclerosis. IMT reflects risk factor burden in children from 10 years of age [274] and thickened IMT has been demonstrated in children with known cardiovascular risk factors such as obesity, diabetes or metabolic syndrome [245, 333, 334]. In non-HIV infected adults, increased IMT is an independent predictor of adverse cardiovascular outcome and a marker of coronary artery disease [290, 335, 336]. HIV infected adults have been shown to have increased IMT compared to matched controls [207] and an increase in IMT over time has been described [337].

I have demonstrated a detrimental (thicker) 0.02mm difference in IMT between HIV infected ART naïve children and age-matched controls and a 0.01mm difference between ART experienced children and age-matched controls. This difference is at the lower end of what other studies have found (differences in mean IMT between HIV

infected and uninfected children ranging from 0.02 to 0.15mm), likely reflecting the younger age and shorter duration of HIV infection in this cohort. Whilst the changes are small and their clinical significance unknown, in the context of ART, when long-term survival is predicted, small changes in vascular function before starting treatment may become very significant over time.

Whilst other studies have shown that IMT increases with age in HIV uninfected children, I did not find any association between age and baseline IMT in either HIV infected or uninfected children. Ishizu et al demonstrated in a group of 60 healthy children aged 5 – 14 years a linear increase in IMT with age [IMT in millimeters = (0.009 x age in years) +0.35] [332]. Charakida et al found an increase of 0.005 mm (95% CI, 0.0003 to 0.01) per additional year of age in HIV infected children but not their controls, in this study the mean age of HIV-infected controls was 11.0 /12.2 years (SD 3.1 / 2.8) [241]. The lack of age effect in the CHAPAS-3 cohort may be a reflection of the young age of the children (only 38% of uninfected controls were >5 years, and 5% > 10 years); it may be that age only starts to impact IMT after the end of the first decade, or that in the older experienced children, whom started ART at an early age (median 3.2 years), this was sufficient to prevent the HIV related increases described by Charakida.

Multivariable regression analysis suggested that in ART naïve HIV infected children and age matched controls, the finding that for each 1mmol/l higher total cholesterol IMT was 0.006mm lower, is at first surprising. When the final model is run replacing total cholesterol with HDL-C very similar results are obtained, such that higher HDL-C is associated with a lower IMT (co-efficient -0.008 95% CI (-0.019, 0 .004)) but this does not reach statistical significance (p=0.19). This is complementary to data from adult cohorts in whom higher HDL-C levels were associated with a thinner IMT [338, 339]. The role of lipids is not straightforward and recent work has shown that interactions between the components of HDL cholesterol is more complex than initially thought and affected by low grade and chronic inflammation [340].

I did not demonstrate an independent association between IMT and absolute CD4 count, CD4 percentage or CD4 z score in either group. In this study a limited set of parameters were studied but further studies looking at naïve/memory ratios and

CD4/CD8 ratios could be informative. In adolescent and adult studies a lower CD4 count and CD4 nadir and in adolescents a lower CD4 nadir is a risk factor for increased IMT although the other variables that were adjusted for differed by study [208, 245, 341]. One possibility is that the CD4 percentage in naïve children does not correlate with baseline IMT as the ratio of naïve to memory cells is altered and it is the ratio that is important.

A trend towards a difference in IMT between ART experienced children and controls (p=0.09) was seen in this CHAPAS-3 cohort but did not reach conventional levels of statistical significance. This is complementary to the findings of the Thai cohort in whom no difference in IMT was described in 100 asymptomatic HIV-infected adolescents who had been on ART (52% on NNRTI based regimen) for a median of 10 years, the majority of whom were virologically suppressed and with a CD4 count above 500cells/uL [240]. In the Thai study, it appears early ART, commenced at a mean age of 3.2 years and continued for a median of 3.9 years, virological suppression, with a CD4≥500 cells/mm3 could be sufficient to reverse/prevent the detrimental changes in IMT reported in other cohorts.

Conversely Sainz et al showed IMT to be higher in a cohort of 150 Spanish HIV infected children compared to 150 controls, 96% of the cohort were on ART although 26% had a detectable VL. Even when removing the patients with a detectable VL a significant difference between HIV+ and HIV- persisted [245]. This may however be a reflection of time with a detectable VL; in the Spanish cohort the median duration of time with a detectable viral load was 10.8 years before ART was commenced so these adolescents were started on ART much later than in the CHAPAS 3 cohort and the Thai cohort [240]. The Spanish cohort also had a large proportion on PIs with a median cumulative exposure of 8.2 years (IQR 3.2 – 11.3 years); a sub analysis of non-smoking vertically infected adolescents in this cohort found PI use to be associated with an increased IMT although no detrimental effects of PI on IMT were seen when the whole cohort was analysed. Finally in the Spanish cohort only a small proportion of children were of African descent (73% white).

This is the first longitudinal cardiovascular data from children living with HIV in Africa. It shows that detrimental changes in IMT can, at least in part, be reversed with ART. Findings that the ART experienced children, who had already been on ART for a mean of 3.9 years, had ongoing improvements in their IMT after a further 2 years ART, suggest a longer duration of ART could continue to be beneficial.

On multivariable analysis the variables found to be associated with significant improvements in IMT were duration of follow up, as discussed above longer ART duration is important and surprisingly baseline urea, in that for every umol/l higher in urea at enrolment a (-)0.01mm greater decrease in IMT was seen over 96 weeks. No association of urea and IMT has been reported previously, this could be reflecting muscle wasting or underlying renal failure, in adult populations an association between renal failure and increased IMT has been reported [342].

Three of the paediatric studies have implicated the use of d4T [243] and protease inhibitors [240, 249] with a thickened IMT when compared to HIV infected adolescents on a regimen containing alternative antiretroviral drugs. All of the 74 ART experienced children were on d4T based treatment at baseline and 22/74 (30%) remained on d4T for the duration of the trial. No difference in IMT was seen between ART experienced children whom remained on d4T or were randomised to ZDV/ABC; similarly in ART naïve children commencing ART there was no difference in the change in IMT over 96 weeks by randomised NRTI arm. Of additional reassurance, given the adverse reports of ABC in the adult population, no difference in IMT was seen in the children randomised to ABC.

5.16.2 PWV results

Arterial stiffness can be an important determinant of the vascular load to the heart and has been associated with adverse cardiovascular outcomes. Aorto-femoral pulse wave velocity (PWV), a measure of arterial elasticity, predicts incident cardiovascular events in asymptomatic adults [335] and is a gold-standard marker of subclinical atherosclerosis [343]. To date little has been published on PWV in vertically HIV-infected children.

I have demonstrated that perinatally HIV-infected ART naïve children have increased arterial stiffness, assessed by measuring PWV, compared to age matched controls, which improves after 2 years of ART. Conversely older children who have been on ART for a median of 3.9 years have similar PWV to healthy HIV uninfected controls but an increase in PWV is seen in the HIV infected children over 2 years follow up; an increase that is not fully explained in terms of increasing age and has been reported in another HIV infected paediatric cohort [242].

In the ART naïve group I found that triglycerides were positively associated with baseline PWV. CD4 percentage and, surprisingly, systolic blood pressure were inversely related with PWV. The later, an inverse relationship between systolic blood pressure and PWV was also seen in the ART naïve children in the original bivariable model (co-efficient -0.012, 95% CI (-0.020, -0.004) p=0.01) suggesting this correlation is real rather than an effect of overcorrecting. In the ART experienced group diastolic blood pressure was positively associated with baseline PWV (co-efficient 0.025, 95% CI (0.011, 0.039) p<0.001).

Charakida et al [242] did not find a difference in PWV between ART naïve and control children, whilst children on ART had a higher PWV than ART naïve despite no significant difference in age, (median age 11 years). There may be some unaccounted for bias as the children with the more severe disease would be treated first and hence had have longer exposure to ART, on multivariate analysis HIV disease severity was an independent predictor of PWV. No significant effect of PI use on PWV was seen in this study.

The importance of early ART is highlighted in a study of South African HIV infected children who were recruited into the CHER study. Innes et al. studied 59 children aged 7 years whom had commenced lopinavir/ritonavir based ART before 3 months of age. 91% had an undetectable VL. Compared to age matched controls there was no difference in PWV (4.8m/sec versus 4.9m/sec p=0.20) after adjustment for age, gender and systolic blood pressure (which was not significantly different between HIV infected children and controls). This was despite significantly elevated lipids in the HIV infected group and prolonged (median 7.1 years) PI based ART [246]. Conversely in a cross

sectional study of 230 HIV infected children aged 6 – 18 years living in Ethiopia, a higher PWV was noted in children on a PI containing regimen [238]. Reassuring I found no difference in change in PWV over 96 weeks between children randomised to d4T, ZDV or ABC.

A further study in HIV infected adolescents has measured PWV in 101 older HIV infected youths (median (IQR) age 19 (14, 23) years) although the majority of this cohort were not vertically infected. Compared to age, sex and race matched controls there was no difference in PWV between HIV infected youths and controls. In the HIV infected group PWV was only associated on multivariate analysis with current alcohol use, although the duration of HIV infection in this study was short (median 8 years). In the control group PWV was positively associated with systolic and diastolic blood pressure, age, BMI, current smoking, marijuana and alcohol use of which age and BMI remained significant on multivariable analysis [247].

An association of higher weight, height and BMI, in HIV uninfected children correlated with an increased IMT has been previously made [240, 249, 344]. Increased body fat accumulation has also been associated with reduced flow mediated dilation [345].

5.16.3 Limitations of the study

One criticism of my study may be the choice of measuring the IMT only in the common carotid artery. Whilst no consensus exists over the gold standard technical protocol for IMT measurement, it can be argued that there are several advantages of taking measurements from the ICA, in that changes may appear here first or be more pronounced [260, 346]. In the only other longitudinal paediatric / adolescent study, significant changes in both the CCA and ICA were observed [249]. From a pragmatic view the training required to reach technical competence for measuring IMT within the ICA would have been far greater and actually getting accurate and reproducible results on children of such a young age whose carotid bulb is higher compared to adults would have been very challenging.

A further limitation is the lack of longitudinal measurements in the HIV-uninfected children. Indeed the natural progression of IMT in healthy young children has not been

studied and whilst improvements in IMT following interventions such as increasing exercise or improved diets have been reported [347-349] it may be that natural changes in IMT are more dynamic than previously thought. In the longitudinal study performed by Ross et al [248] a decrease in the IMT (measured in the ICA) was seen over 48 weeks in the control group despite no interventions, suggesting there may be normal fluctuations in cIMT among this age group (median age 10 years). The lack of normal ranges in this age range and in African children makes prediction of “normality” difficult. Future work needs to focus on the natural progression of IMT and PWV in HIV uninfected African children. Secondly the natural progression of IMT and PWV changes in HIV infected, ART naïve children who are not started on ART is not known. Given that since 2013 WHO has recommended that all children under 5 years should be on ART as soon as a diagnosis is made it is unlikely that the longitudinal effects of untreated paediatric HIV on the cardiovascular system will ever be able to be determined.

Being overweight causes adverse changes in the metabolic profile, insulin resistance and full metabolic syndrome can develop: increased IMT and PWV are well described in such patients. In our cohort the other end of the spectrum – malnutrition – was common and to date the limited literature that exists suggests adult survivors of malnutrition have lower PWV and IMT compared to adults who have not suffered from malnutrition [350]. Equally low birth weight and rapid weight gain in early childhood has been proposed to increase the risk of later cardiovascular disease. Unfortunately although we tried to collect data about gestational age at delivery and birth weight this information was generally not available in either the HIV infected children or uninfected controls. In a study of 19 year olds who had been born prematurely, IMT was unrelated to gestational age, birth weight standard deviation score and early postnatal weight gain but was positively associated with late infancy weight gain, although the relationship disappeared after correction for current height [351].

Contradictory results to the CHAPAS-3 from previous studies may reflect the smaller sample size in many of these other studies, study design (heterogeneous ART exposure), limited follow up and lack of comparability of controls. By limiting

confounders, for example smoking or obesity, it has been possible to demonstrate the impact of HIV disease and ART treatment. The CHAPAS 3 data supports a role for HIV infection per se rather than for HIV related factors or ART as suggested by other studies [241, 249]. One theory postulated is that chronic HIV infection leads to vascular endothelial damage, ongoing low level sustained inflammation leading to an increase in vessel wall thickness [352]. The CHAPAS-3 findings suggest that uncontrolled HIV infection plays a major role in the pathogenesis of the cardiovascular changes. In chapter 6 this will be explored in more detail.

The CHAPAS 3 study has several advantages. It is the first to study a significant proportion of very young children. Secondly the control group was carefully matched for age, sex and ethnicity. However of the control children a proportion were uninfected ART exposed children (absolute number not collected) and the influence of in utero ART exposure to cardiovascular parameters remains to be fully determined.

5.17 Conclusions

From a very young age detrimental changes in markers of cardiovascular structure and arterial stiffness are seen in untreated HIV infected children living in Zambia and Uganda. Using ART to suppress viral replication and inflammation, while improving immune function appears to be protective and can reverse some of the detrimental atherogenic structural and functional changes. No differential effects of three different NRTI backbones were demonstrated on the effect on the cardiovascular system. With long term survival expected for vertically infected infants and children this evidence adds to the known benefits of ART. Longitudinal monitoring of cardiovascular structure and arterial stiffness needs to be undertaken, especially looking at the effects of newer ART regimens on cardiovascular structure and arterial stiffness.

Chapter 6 Results: The impact of ART on markers of inflammation, vascular dysfunction and disordered thrombogenesis

6.1 Introduction

Adverse cardiovascular outcomes in HIV infection and potential underlying mechanisms have been described in chapter 2. In summary, despite extensive investigation, the pathophysiology underlying the changes in vascular function associated with HIV infections have yet to be fully elucidated. Important factors include, increased inflammation, classical cardiovascular risk factors (although in children the traditional risk factors are unlikely to be operating by themselves, if at all), disordered vascular repair mechanisms and ART-related metabolic toxicities. Inflammation may be key, as evidenced by increased levels of pro-inflammatory cytokines such as IL-6 and CRP, even when virological suppression is achieved and maintained [80]. For this study 19 biomarkers were selected that have been associated with vascular diseases. These were measured in the CHPAS 3 cohort and then analysed in the context of a range of parameters that could be important in HIV and vascular pathogenesis. The majority of available evidence is derived from adult studies. Paediatric data is sparse and contradictory. Table 6-1 summarises the key actions of each of the 19 biomarkers, the effects of HIV on the biomarkers and known associations with cardiovascular disease in adults.

Number recruited	Setting	% patients on ART	Parameters measured	Findings	Ref
83 HIV + 59 HIV – Aged 5.4 – 17.7 years	UK.	33% ART naïve 67% on ART *(31/56 PI 25/56 non PI)*	cIMT, FMD, BP, anthropometry, total cholesterol, HDL, apoB, LPa, LDL, hsCRP	Lipid abnormality not associated with IMT / FMD. Increased hsCRP in HIV +	[241]
64 HIV + 30 HIV – mean age 14.1 years	Spain	All on ART, 83% with VL <50 copies/mL	IMT, IL-6, CRP. MPO. tPA, sVCAM-1, P-selectin, sCD14, lipid profile	HIV + higher sCD14, sVCAM. No relationship between biomarkers and VL. No effect HIV status for CRP, IL-6, MPO, MCP-1, P-selectin and tPA. No correlation between biomarkers and IMT.	[244]
77 HIV + 32 HV – 13% horizontally infected Median age 16.2 years	Spain	70% with undetectable VL 30% with VL > 50	hsCRP, D-dimer, β2-microglobulin, HLA-DR+CD38+, LPS, microbial 16srDNA, sCD14	HIV+ higher CRP, d-dimer, β2microglobulin; no ART class effect. HLA DR+CD38+ cells increased in viraemic children. Higher levels of LPS, 16s RNA and sCD14 independent of viraemia.	[439]
31 HIV+ stable on ART for >6 months 31 HIV -	USA	84% VL < 400 copies/ml 16/31on PI based ART	cIMT, homocysteine, hsCRP, myeloperoxidase, lipids	HIV+ higher cholesterol, triglycerides, MPO and lower homocysteine levels. BMI significant predictor of IMT in HIV-, duration of ART predictive of IMT in HIV+	[249]
106 HIV+, mean age 14.8 years, 5% horizontally infected 55 HIV -, mean age 12.3 years	USA	86% on ART	CRP, IL-6, MCP-1, fibrinogen, P-selectin, sICAM< sVCAM, E-selectin, leptin, anthropometry	HIV+; higher sICAM, sVCAM, MCP-1, IL-6, fibrinogen. Higher IL-6, MCP-1, CRP and sICAM correlated with higher waist:hip, sVCAM, MCP-1, IL-6, fibrinogen and CRP inversely related to CD4%.	[440]

Table 6-1. Markers of inflammation and vascular injury tested in this study

	Actions	**Effect of HIV infection**	**Association with cardiovascular disease**
Markers of inflammation			
IL-6	Pro and anti-inflammatory actions Secreted early in the inflammatory pathway, stimulates acute phase protein synthesis, neutrophil production, supports B cell development Antagonistic actions against regulatory T cells, inhibits TNFa / IL-1 activates IL-1Ra / IL-10	Higher levels in HIV+ adults that do not normalise with ART / viral load suppression [78, 80, 353-355] Higher levels related to increased risk of opportunistic infections [356], disease progression [357] non-AIDS defining events [316] and all-cause mortality [78, 358, 359]. Higher levels in obese HIV+ adults [360] Conflicting impact of ART e.g. current ABC use; no effect on IL-6 [176] or higher IL-6 levels [151]	HIV+: Higher levels associated with CVD [3] conversely in a cohort study no significant difference between cases and controls [361] HIV-: Higher levels associated with CVD, endothelial dysfunction [362], increased risk of coronary events [363, 364] and subclinical atherosclerotic lesions independent of traditional risk factors [365-367]
IL-8	Produced by macrophages and epithelial cells A potent chemotactic factor for granulocytes and T cells to phagocytose antigens which trigger toll-like receptors.	Higher levels in HIV+ adults unrelated to disease activity, co-infections or other medications [368]. A small study failed to demonstrate an impact of HIV status on IL-8 levels, however levels were higher with active TB co-infection [369].	Limited in vitro evidence suggestive a link between higher levels and increased CVD risk but minimal in vivo evidence [370]

	Actions	Effect of HIV infection	Association with cardiovascular disease
IL-10	Anti-inflammatory and anti-atherosclerotic cytokine produced by monocytes and lymphocytes Inhibits synthesis of pro-inflammatory cytokines including TNFα and suppresses the antigen presentation capacity of antigen presenting cells Enhances B cell survival, proliferation, and antibody production	Conflicting results; Higher levels in HIV+, increase with disease progression and fall with ART but not to normal levels [371]. Levels correlate with viral load [372] and inversely correlate to CD4 [373] Conversely a small study showed that levels increase after starting ART and fall following treatment interruption [374]	No relation demonstrated with CV events [361], lower levels associated with increased cardiovascular risk [375] and thickened IMT [376]
TNFα	Produced by activated macrophages, lymphoid cells, endothelial cells, cardiac myocytes and adipose tissue. Stimulates the acute phase reaction, promotes expression of adhesion molecules on endothelial cells and inhibits viral replication. A potent chemo-attractant for neutrophils, stimulates macrophage phagocytosis and production of IL-1. Alters intracellular calcium homeostasis and induces nitric oxide synthesis which can injure myocardial cells	Higher levels early in HIV infection and correlate with viral load [372], disease progression [377], being overweight [10], poor prognosis [378] and ART failure [379]. Levels remain elevated despite effective ART [354, 379-382] - not fully understood but reduction with effective ART reported in a small study [383]	HIV+: adults on ART higher levels related to increased coronary arterial calcification [384] HIV-: Higher levels in patients with abnormal ECG or symptomatic heart disease [364].
IL-1Ra	Inhibits the pro-inflammatory actions of IL-1, IL-1α and IL-1β, secreted by epithelial cells and adipocytes	Significantly elevated in HIV+ unaffected by disease stage or CD4 cell count [385] or inversely related to CD4 count [373]	Early release at sites of ruptured plaque, elevated following myocardial infarction [386]

	Actions	Effect of HIV infection	Association with cardiovascular disease
CRP	A pro-inflammatory acute-phase reactant stimulated by IL-6 Produced mainly by the liver and within atherosclerotic plaques by activated vascular cells Induces adhesion molecule expression in human endothelial cells, may play a direct role in promoting the inflammatory component of atherosclerosis [387]	Higher levels associated with increased BMI [360], dyslipidaemia [388], male sex, opportunistic infections [356], cardiovascular disease [389] and all-cause mortality [78, 390-393]. No association with ART, viral load or CD4 count [80, 394]. Conflicting effect of ART - ABC use no difference [176] or raised levels seen[151] Persistently elevated CRP with suppressed VL associated with clinical failure [395]	HIV+ adults higher levels associated with increased risk of coronary events [363, 396-398], adverse IMT changes [84, 354, 366], measuring CRP helps identify those at high risk of CVD [399] HIV- adults elevated levels an independent factor associated with CVD [400-402].
ICAM-3 (CD50)	A transmembrane glycoprotein that binds a leukocyte adhesion protein (LFA-1), an important step in the initiation of the immune response.	No associations found	No associations found
Serum amyloid A	An acute phase protein produced by hepatocytes and adipocytes, that assists cell recruitment to the sites of inflammation and induces enzymes that degrade the extracellular matrix.	A non-significant increase in HIV-infected patients who have died [78]	Increased in HIV+ patients whom had a cardiovascular event compared to HIV infected patients with no history of a cardiovascular event [361]

	Actions	Effect of HIV infection	Association with cardiovascular disease
Markers of vascular injury			
E-selectin (CD62E)	A cell adhesion molecule expressed by activated endothelial cells which has a pro-inflammatory role and involved in the pathogenesis of atherosclerosis [403]	Conflicting results - levels higher in HIV+ adults and correlate with disease progression, levels increased in seronegative controls with bacterial and protozoal infections [404]. No difference from controls [82]	Higher levels seen in patients with CHD and independently predict risk of CHD [405]
P-selectin	Cell adhesion molecule stored in endothelial cells and platelet granules that promotes leucocyte adherence to the endothelium A marker of platelet activation	Levels fall following initiation of ART [374] although another study suggested elevated levels on ART related to dyslipidaemia [406, 407].	Elevated levels proposed as a strong correlate of platelet/endothelial abnormalities in patients with major atherosclerotic risk factors [408]
sVCAM1 (CD106)	Adhesion molecule released in endothelial dysfunction and activation Stimulated by TNFα and IL-1 Has a role in inflammatory component of atherosclerosis [403]	Higher levels in HIV+ [409]; not associated with disease stage [354, 410] nor related to CD4 or CRP [82], higher levels if have lipodystrophy [411] Levels fall with starting ART and increase following treatment interruption [374, 412, 413].	HIV+ levels increased up to 2 years before a cardiovascular event [361] In HIV- population no association with higher levels and future risk of MI [405, 414] .
ICAM-1 (CD54)	An endothelial and leukocyte-associated transmembrane protein stimulated by IL-1 and TNFα. Important in stabilizing cell-cell interactions, facilitating leukocyte endothelial transmigration and in the inflammatory component of atherosclerosis [403]	Higher in HIV+ [415, 416], in patients with advanced disease and proportional to CD4 count [410], not related to CRP levels [82]. Levels increase following treatment interruption [412] Higher levels in ART naïve which normalized with treatment but not to normal levels[417], levels related to serum lipid levels [406].	In HIV+ levels are not related to CV events[361] In HIV- higher levels associated with presence of atherosclerotic plaques [365] and independently predict risk of CHD independent of other known risk factors [405]

	Actions	Effect of HIV infection	Association with cardiovascular disease
MCP-1	Pro-inflammatory Recruits monocytes, memory T cells, and dendritic cells to sites of tissue injury, infection and inflammation. Implicated in the pathogenesis of atherosclerosis	Higher levels seen in HIV infection [418], TB co-infection [369] and treatment interruption [413, 419]. Levels related to degree of lipodystrophy [420] and inversely related to CD4 [373]. Higher CSF levels correlate with HIV associated dementia [421].	In HIV+, higher levels seen with increased IMT [420, 422], pre-atherosclerotic changes [418] and increased coronary calcification [384] HIV-: higher levels correlate with subclinical atherosclerosis [423], significant associations with age, smoking status, and serum triglyceride concentrations [423]
VEGF	A signal protein that stimulates vasculogenesis and angiogenesis	Serum levels increased in HIV-associated neurological disease [424], higher levels with higher CD4 counts, implicated in pathogenesis of Kaposi's sarcoma [425]	Unclear
Angio-poietin	A protein growth factor that promotes angiogenesis,	Implicated in pathogenesis of Kaposi's sarcoma [426]. An increase in the ANG-2:ANG-1 ratio is observed during acute HIV infection and ANG-1 falls with chronic HIV infection [427]	Disequilibrium of ratio described in cardiac failure [428]

	Actions	Effect of HIV infection	Association with cardiovascular disease
Markers of disordered thrombogenesis			
Tissue factor (CD142)	A glycoprotein expressed on vascular smooth muscle cells and on atherosclerotic plaques Primary initiator of the extrinsic coagulation pathway leading to conversion of prothrombin to thrombin and fibrin clot formation. . Involved in non-haemoastatic processes e.g. inflammation, angiogenesis, tumour growth and metastasis.	Increased expression on monocytes correlated, levels correlated with VL and indices of immune activation, [429]	Elevated levels seen in HIV- patients with cardiovascular risk factors such as hypertension, diabetes, dyslipidemia, and smoking as well as in those with acute coronary syndromes [430]
d-dimer	Fibrin degradation products, a specific marker of fibrin clot formation and fibrinolysis	Higher levels with higher VL [80, 415], advancing age [357, 381], severe immunosuppression, inflammatory disease or malignancy [431]. Higher levels related to all-cause mortality [78, 316] but only modestly affects risk reclassification [88] Higher levels in ART naïve, normalized with treatment but not to normal levels [374, 417, 432], No effect of ABC [176]	HIV+, higher levels independently associated with increased risk of CVD [361, 389] HIV-, higher levels are associated with an increased risk of MI but this is not an independent risk [433]
thrombo-modulin (CD141)	An integral membrane protein expressed on endothelial cells which functions as a cofactor in the thrombin-induced activation of protein C in the anticoagulant pathway	Elevated in HIV+ ART naive, non-significant reduction with ART, significant increase in levels significantly higher if have peripheral lipoatrophy [411]	Preliminary results suggest that thrombomodulin dysfunction may be implicated in the pathogenesis of myocardial infarction [434]

6.2 Methods

An extensive panel of 19 biomarkers, reflecting markers of inflammation, disordered thrombogenesis and vascular injury and repair was performed on all samples (HIV infected and controls) at baseline and in the HIV infected children at weeks 48 and 96. Methods of the techniques used are described in chapter 3.

6.2.1 Analysis

As expected, the absolute biomarker concentrations were highly skewed. Whilst I can compare the different groups using non-parametric rank-sum tests, these have lower power than parametric tests (like the t-test). Further, I wish to investigate the effects of different baseline factors on the biomarkers which require a regression model. To use a standard normal linear regression, the outcome (dependent) variable needs to be approximately normally distributed. I used a "boxcox" regression to estimate the best power transformation to obtain approximate normality. This suggested that a log transformation was the closest simple power transformation for a regression model: I used log10 since this is what is commonly used for HIV viral load. 16/19 biomarkers were truncated at the 99th percentiles, and IL-6, IL-8 and TNFa were truncated at the 95th percentile, to avoid outliers having undue influence on results. A small number of results below the limits of detection were set to the lower limit of detection of each assay (8/4 tissue factor from (control) groups 3/4 respectively, and 8/15/5/10 d-dimer from groups 1/2/3/4 respectively). Where VL was <100 copies/ml, a value of 100 copies/ml was used for analysis; effectively therefore this factor measures the impact of log10 increases above detectable with undetectable as the reference category. Z scores for weight/height/BMI for age were obtained from UK 1990 cohort data [435], WHO z-scores were not used as these do not cover the age range of CHAPAS-3 children for weight. Z scores for CD4 were obtained from [436].

6.3 Results

Reproducibility of the Assays

Between 43 and 57 (20%-28%) of baseline samples were run in duplicate to ensure reproducibility between plates. Where samples were run twice the results from the first run were used for the analysis. Results from the second run were used for the purpose of validation only.

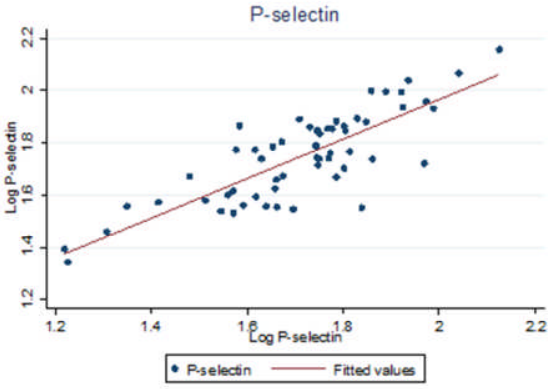
Bland Altman plots were constructed for each biomarker and the mean difference, confidence intervals, limits of agreement and difference in variance calculated for each marker, Table 6-2, Figure 6-1. For nine of the nineteen biomarkers (P-selectin, ICAM-3, thrombomodulin, Ang-2, IL-1Ra, IL-10, IL-6, MCP-1 and VEGF) the mean difference between duplicates was significantly different from zero (Bland Altman plots shown below Table 6.1). However the mean differences were relatively small, compared to the spread of the data, with limits of agreement within +/- 0.4 except for IL-10 and IL-8 which were within +/- 0.45, and IL-6, CRP and VEGF which were within +/- 0.6. Further there was no evidence of systematically poorer agreement at low or high values, suggesting that the values have more random variation in observed values across the data range. Therefore using one measurement per sample would increase the random error, and reduce my ability to detect genuine associations, but should not to lead to bias. I was not able to run all assays in duplicate due to funding constraints.

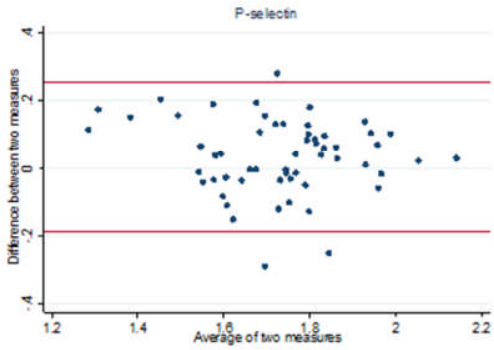
Table 6-2. Summary of results from biomarker validation study.

Mean difference (log10) and 95% confidence intervals given

Biomarker	Number tested	Mean difference	95% CI		Limits of agreement	
E-selectin	57	0.00	-0.05	0.04	-0.34	0.34
P-selectin	57	0.04	0.01	0.07	-0.18	0.26
ICAM-3	43	-0.05	-0.09	-0.01	-0.28	0.18
Thromb	57	0.04	0.02	0.06	-0.13	0.22
Ang-1	57	-0.01	-0.03	0.01	-0.15	0.13
Ang-2	43	-0.11	-0.15	-0.07	-0.37	0.16
DD	43	0.01	-0.03	0.05	-0.20	0.22
TF	43	0.03	-0.02	0.09	-0.20	0.26
IL1-RA	43	0.03	0.01	0.05	-0.09	0.15
IL-10	57	-0.09	-0.14	-0.05	-0.44	0.25
IL-6	43	-0.14	-0.20	-0.07	-0.53	0.26
IL-8	57	-0.03	-0.08	0.02	-0.44	0.38
MCP-1	43	-0.03	-0.05	-0.01	-0.14	0.08
TNFa	57	-0.02	0.00	0.04	-0.15	0.19
CRP	57	0.01	-0.08	0.10	-0.61	0.63
SAA	43	-0.02	-0.07	0.04	-0.35	0.32
SICAM1	57	0.00	-0.06	0.05	-0.39	0.38
VCAM-1	57	-0.02	-0.07	0.03	-0.36	0.31
VEGF	57	-0.12	-0.19	-0.05	-0.63	0.40

IL-1Ra, interleukin-1 receptor antagonist; CRP, C reactive protein; TNFa, tumour necrosis factor A; IL-10, interleukin-10; IL-6, interleukin-6; IL-8, interleukin-8; ICAM-3, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; Angio 1, angiopoietin-1; Angio 2, angiopoietin-2, Ang 2: Ang 1, ratio of angiopoietin 2:1; sICAM, soluble intracellular adhesion molecule; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; TF, tissue factor; TM, thrombomodulin.


P-selectin
Log P-selectin
Log P-selectin
P-selectin
Fitted values


P-selectin
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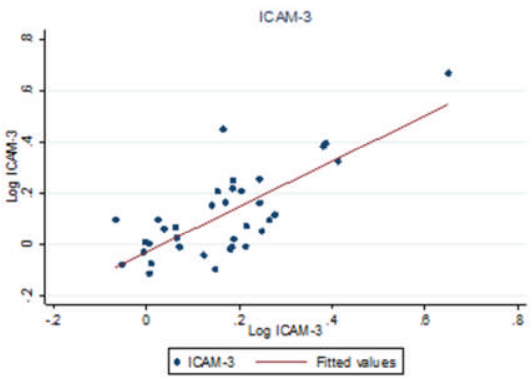
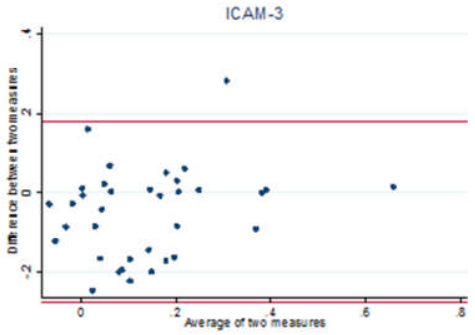
Average of two measures


ICAM-3
Log ICAM-3
Log ICAM-3
ICAM-3
Fitted values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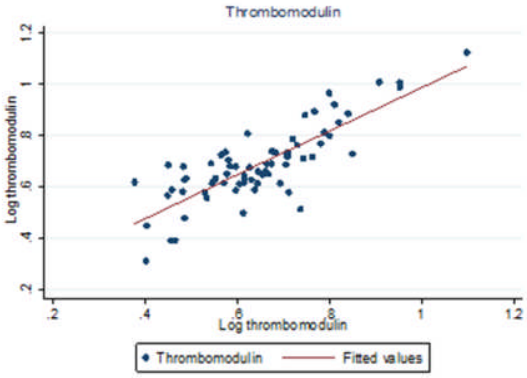
Average of two measures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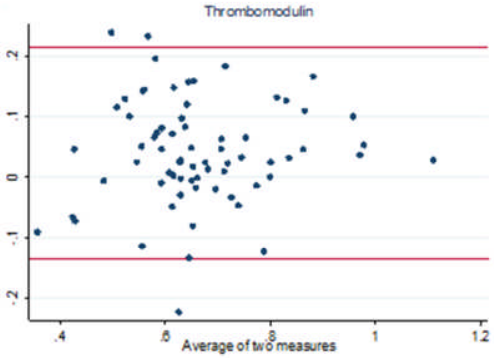
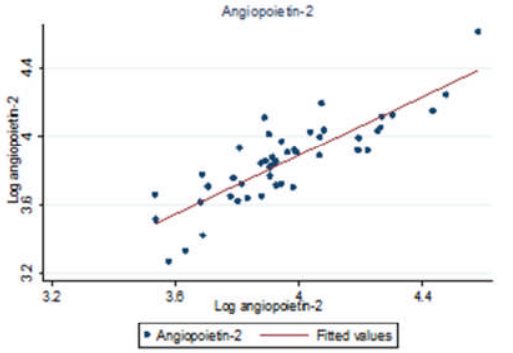
Log thrombomodulin
Log thrombomodulin
Thrombomodulin
Fitted values


Thrombomodulin
Average of two measures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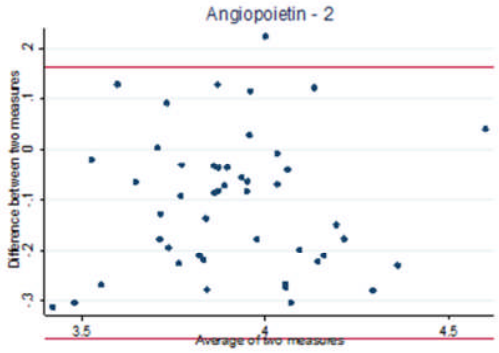
Log angiopoietin-2
Angiopoietin-2
Fitted values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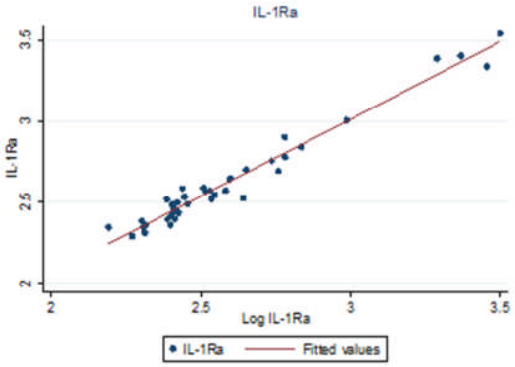
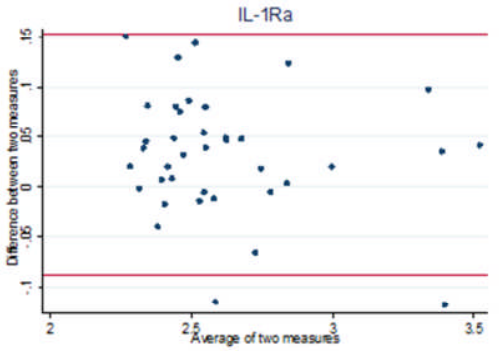
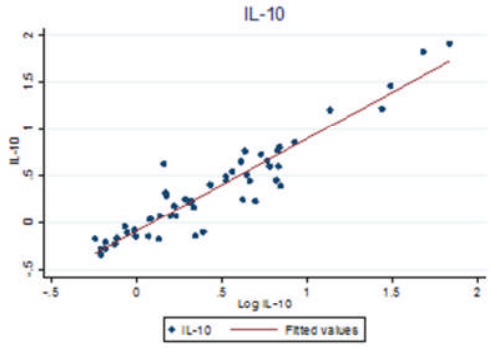
Average of two measures


IL-1Ra
IL-1Ra
Log IL-1Ra
IL-1Ra
Fitted values


IL-1Ra
Difference between two measures
Average of two measures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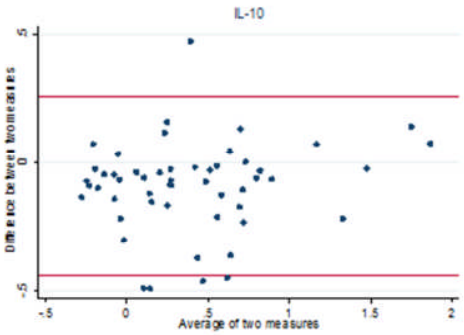
IL-10
Log IL-10
IL-10
Fitted values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Average of two measures

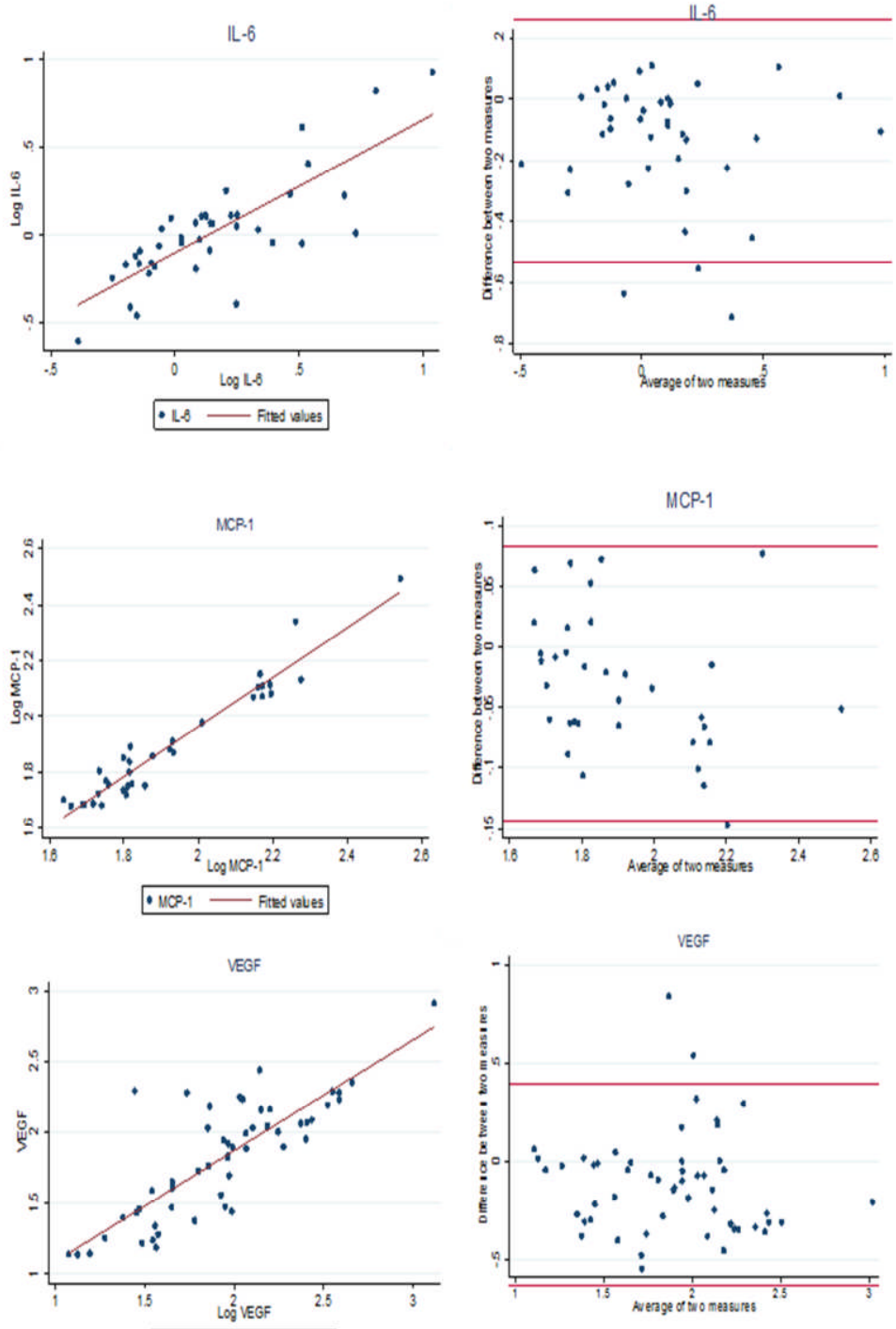


Figure 6-1. Results of the biomarker validation study.

Scatter plots to show correlation and limits of agreement for nine of the nineteen biomarkers.

6.3.1 Missing samples

As summarised in Table 6-3, the majority of patients had blood samples available at each time point. A few patients did not have samples available due either to difficulties with venesection or mislabeling of samples. All analyses are based on observed data.

Table 6-3. Summary of blood samples available by week.

		Group 1 HIV infected ART naïve	HIV uninfected matched to group 1	Group 2 HIV infected ART experienced	HIV uninfected matched to group 2
Week 0	Total	208	209	74	75
	Samples available	205	202	73	73
	Missing (%)	3 (1)	7 (3)	1 (1)	2 (3)
Week 48	Total	185		74	
	Samples available	185		73	
	Missing	0		1 (1)	
Week 96	Total	178		74	
	Samples available	174		74	
	Missing	4 (2)			
Week 144	Total	31		21	
	Samples available	31		21	
	Missing	0		0	

6.4 Baseline biomarker results

Baseline results are summarised in Table 6-4. In summary:

Inflammatory markers (IL-1Ra, CRP, TNFa, IL-10, IL-6, IL-8, ICAM-3, SAA)

IL-1Ra and IL-10 have anti-inflammatory actions; levels of both were significantly higher in the ART naïve children compared to their controls and compared to ART experienced children ($p<0.001$). IL-1Ra levels in ART experienced children and their controls did not differ significantly ($p=0.49$) whilst IL-10 levels were significantly higher in ART experienced children compared to their controls ($p<0.001$)

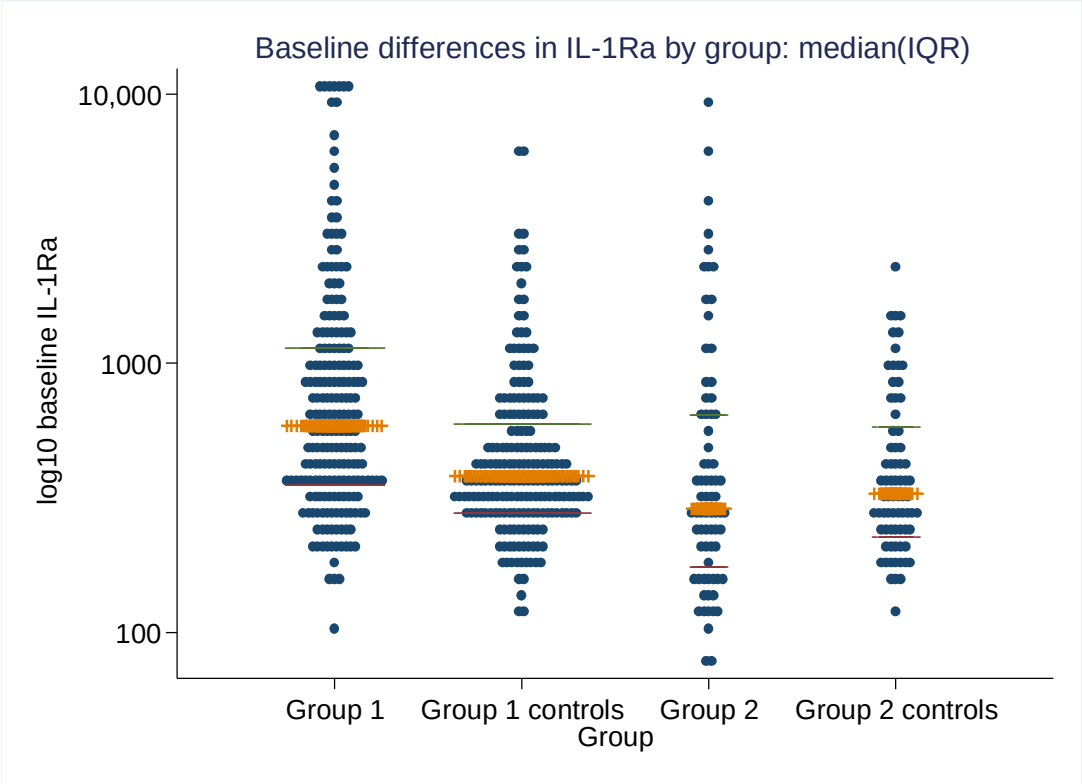


Figure 6-2. Baseline differences between Il-1Ra between ART naïve (group 1) and controls and ART experienced (group 2) and controls. Median and IQR given.

6 pro-inflammatory markers (CRP, TNFa, IL-6, IL-8, ICAM-3, SAA) were measured. Comparing ART naïve children to controls all 6 pro-inflammatory markers were significantly higher in the ART naïve children ($p<0.001$). All 6 biomarkers were also significantly higher in ART naïve children compared to the ART experienced group ($p\leq0.008$). Comparing ART experienced children to their control group CRP, IL-6, IL-8 and ICAM-3 levels were higher in the ART experienced group ($p\leq0.04$). TNFa levels were significantly higher in the control group compared to the ART experienced group ($p=0.02$) (although absolute differences were relatively small) whilst SAA levels were comparable between ART experienced and their controls ($p=0.61$), Figure 6-3.

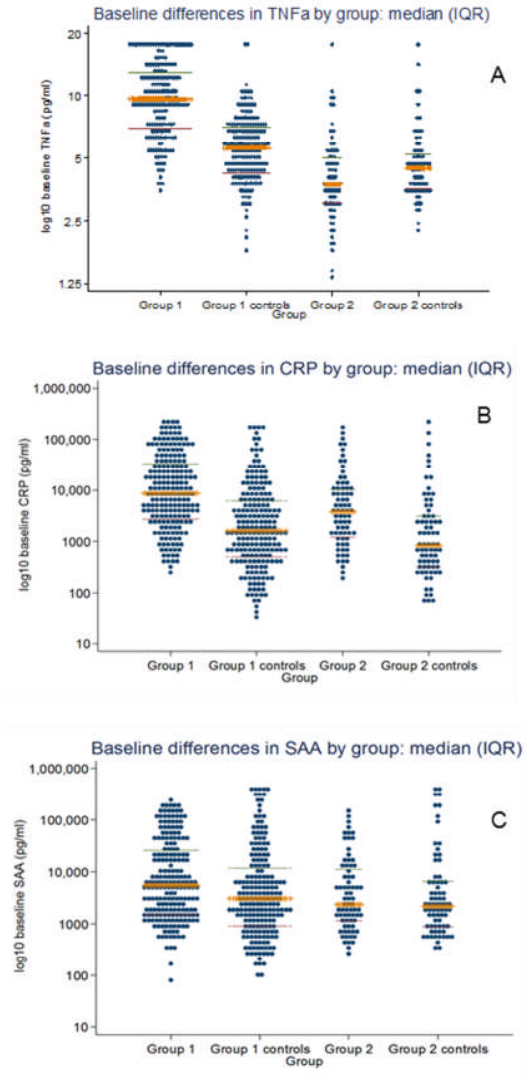


Figure 6-3. Examples of the baseline differences between 3 pro-inflammatory biomarkers.

A) TNFa levels were significantly higher in ART naïve children compared to their controls and compared to ART experienced children. However levels in ART experienced children were significantly lower than their controls.

B) CRP levels were significantly higher in ART naïve children compared to controls and ART experienced children. ART experienced children also had significantly higher levels then their controls

C) Serum amyloid A levels were significantly higher in ART naïve compared to controls and ART experienced children, however levels in ART experienced and their controls were similar.

Markers of cardiovascular injury and repair (MCP-1, Ang-1, Ang-2, E-selectin, P-selectin, sICAM-1, VCAM, VEGF)

Comparing ART naïve children to controls, with the exception of Ang-2, all markers of cardiovascular injury were higher in ART naïve children ($p \leq 0.03$). Ang-2 levels were significantly higher in the control group compared to the ART naïve group ($p < 0.001$). Comparing ART naïve to ART experienced children 7 of the 8 cardiovascular markers were higher in ART naïve children; Ang-1 levels were comparable between these groups. Comparing ART experienced children to controls, 5 of the 8 markers of cardiovascular injury were higher in ART experienced children. Levels of Ang-2 and E-selectin were higher in controls compared to the ART experienced children ($p \leq 0.002$) whilst sICAM-1 levels were similar between ART experienced and control children. Examples are given Figure 6-4.

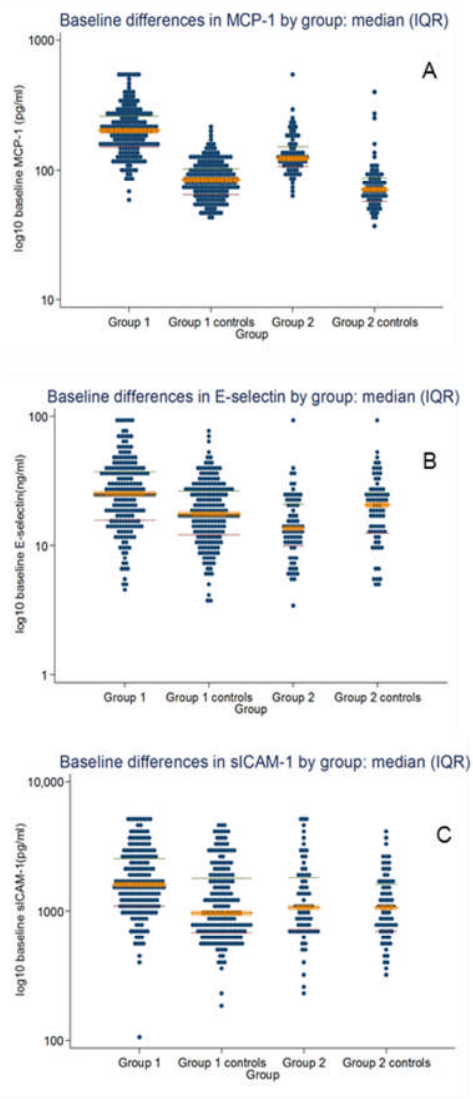


Figure 6-4. Examples of the baseline differences between 3 markers of cardiovascular injury and repair.

A) MCP-1 levels were significantly higher in ART naïve children compared to their controls and compared to ART experienced children. ART experienced children also had significantly higher levels than their controls.

B) E-selectin levels were significantly higher in ART naïve children compared to controls and ART experienced children. ART experienced children had significantly lower levels then their controls

C) Soluble ICAM-1 levels were significantly higher in ART naïve compared to controls and ART experienced children, however levels in ART experienced and HIV uninfected children were similar.

As a consequence of higher Ang-1 and lower Ang-2, the Ang2:Ang-1 ratio was significantly lower in ART naïve children compared to controls and in ART experienced children compared to controls. The ratio was higher in ART naïve children compared to ART experienced children - Figure 6-5.

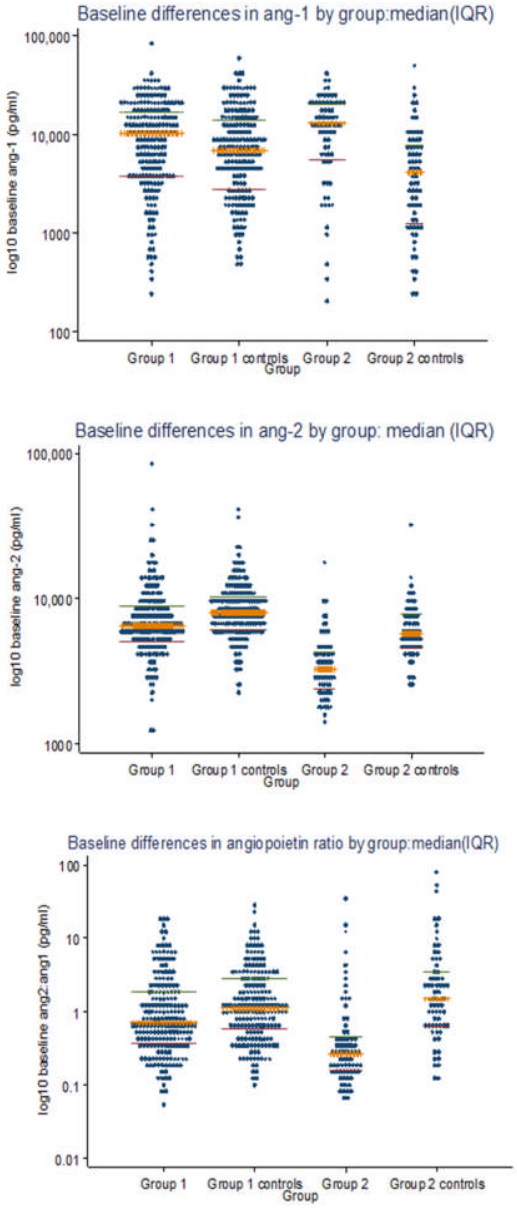


Figure 6-5. Baseline differences in Ang-1, Ang-2 and Angiopoietin 2:1 ratio between ART naïve, ART experienced and controls.

Markers of disordered thrombogenesis (d-dimer, TF, TM):

Comparing ART naïve children to controls, D-dimer levels were significantly higher in the ART naïve group ($p<0.001$) whilst thrombomodulin (TM) and tissue factor (TF) levels were more similar, although there was a trend to marginally lower thrombomodulin in controls ($p=0.08$). Comparing ART naïve to ART experienced D-dimer and TM levels were higher in the ART naïve group ($p<0.001$), conversely TF levels were higher in the ART experienced group ($p=0.003$). Levels of D-dimer and TF were higher in the ART experienced group compared to controls ($p<0.001$). Levels of TM were higher in controls compared to the ART experienced children ($p<0.001$), Figure 6-6.

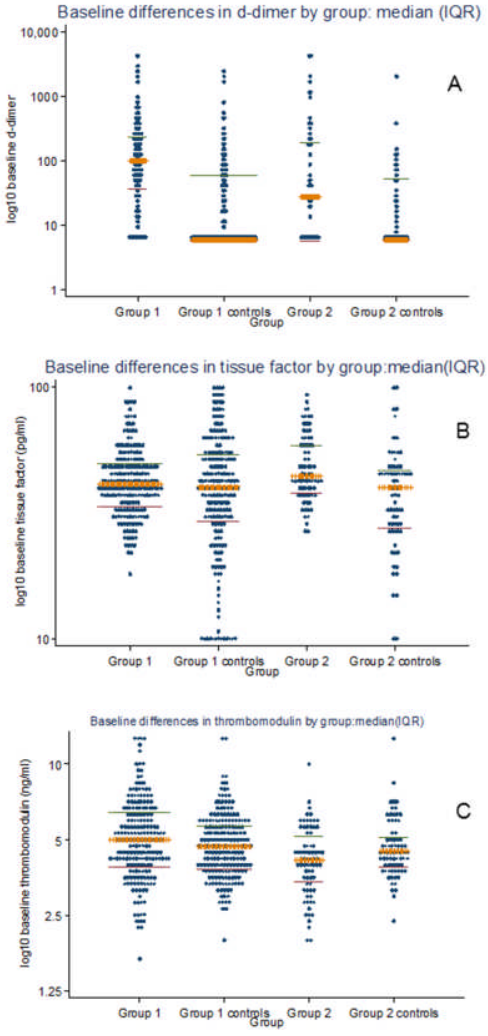


Figure 6-6. Examples of the baseline differences between the 3 markers of disordered thrombogenesis.

A) D-dimer levels were significantly higher in ART naïve children compared to their controls and compared to ART experienced children. Levels in ART experienced children were also significantly higher than their controls.

B) Tissue factor levels were comparable between ART naïve children and their controls. Levels in ART experienced children were significantly higher levels than their controls and ART naïve children

C) Thrombomodulin levels were significantly higher in ART naïve compared to ART experienced children but there was only a marginal trend towards a difference between ART naïve children and their controls (p=0.08). ART experienced children had higher levels than their controls.

Table 6-4. Summary of baseline biomarker results by group.

	HIV infected ART naïve (Group 1)				HIV uninfected matched to group 1				difference between group 1 and controls*
	No	Median	IQR		No	Median	IQR		
Inflammatory									
IL-1Ra	205	586	357	1150	202	381	283	604	**p < 0.001**
CRP	205	8675	2823	34410	202	1623	533	6461	**p < 0.001**
TNFa	205	9.6	7.1	13.0	202	5.6	4.2	7.1	**p < 0.001**
IL-10	205	7.8	4.7	18.0	202	1.8	1.3	2.8	**p < 0.001**
IL-6	205	11.9	7.0	20.8	202	0.9	0.6	1.6	**p < 0.001**
IL-8	205	12.1	5.7	27.3	202	4.3	2.3	7.0	**p < 0.001**
ICAM-3	204	11.9	8.6	16.1	199	1.4	1.1	1.6	**p < 0.001**
SAA	205	5583	1592	27357	202	3110	966	12529	**p < 0.001**
Cardiovascular injury and repair									
MCP	205	203	155	267	202	85	66	105	**p < 0.001**
Angio 1	197	10579	4011	17581	187	7066	2881	14580	**p = 0.03**
Angio 2	197	6485	5120	9039	188	8023	6199	10426	**p < 0.001**
Ang 2:Ang 1	197	0.72	0.39	1.86	187	1.12	0.59	2.92	**p = 0.001**
E-selectin	204	25.3	16.1	38.2	199	17.6	12.4	27.0	**p < 0.001**
P-selectin	204	78.4	54.2	114.9	199	51.7	40.1	67.4	**p < 0.001**
sICAM	205	1612	1113	2602	202	966	695	1839	**p < 0.001**
VCAM	205	2549	1820	4009	202	1056	710	1751	**p < 0.001**
VEGF	205	356	158	725	202	66	36	118	**p < 0.001**
Disordered thrombogenesis									
D-dimer	204	101	37	247	200	5.9	5.9	61.7	**p < 0.001**
TF	203	41.1	33.6	49.7	199	40	29	54	p = 0.34
TM	204	5.0	3.9	6.4	199	4.7	3.8	5.7	p = 0.08

** using Mann-Whitney U test; IL-1Ra, interleukin-1 receptor antagonist; CRP, C reactive protein; TNFa, tumour necrosis factor A; IL-10, interleukin-10; IL-6, interleukin-6; IL-8, interleukin-8; ICAM-3, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; Angio 1, angiopoietin-1; Angio 2, angiopoietin-2, Ang 2: Ang 1, ratio of angiopoietin 2:1; sICAM, soluble intracellular adhesion molecule; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; TF, tissue factor; TM, thrombomodulin.*

Significantly higher in ART-naïve vs controls, ART-experienced vs controls and ART-naïve vs experienced

Significantly higher in ART-naïve vs controls and vs ART-experienced; no evidence of difference between ART-experienced vs controls

Significantly (or marginally) higher in ART-naïve vs controls and vs ART-experienced; significantly lower in ART-experienced vs controls

Significantly lower in ART-naïve vs controls and in ART-experienced vs controls; significantly higher in ART-naïve vs experienced

Note: patterns seen in >1 biomarker indicated by shading

	HIV infected ART experienced (Group 2)				HIV uninfected matched to group 2				difference between group 2 and controls*	Difference between Group 1 v Group 2*
	No	Median	IQR		No	Median	IQR			
Inflammatory										
IL-1Ra	73	289	178	652	73	328	230	589	p = 0.49	**p < 0.001**
CRP	73	3791	1283	10781	73	830	333	3283	**p < 0.001**	**p < 0.001**
TNFa	73	3.7	3.1	5.1	73	4.5	3.6	5.3	**p = 0.02**	**p < 0.001**
IL-10	73	2.7	1.8	7.2	73	1.4	1.0	2.4	**p < 0.001**	**p < 0.001**
IL-6	73	6.4	4.6	10.3	73	0.8	0.6	1.5	**p < 0.001**	**p < 0.001**
IL-8	73	6.8	3.1	11.6	73	3.4	2.0	8.7	**p = 0.04**	**p < 0.001**
ICAM-3	73	4.4	3.4	5.3	73	1.5	1.2	1.8	**p < 0.001**	**p < 0.001**
SAA	73	2368	1207	11843	73	2172	933	6856	p = 0.61	**P = 0.008**
Cardiovascular injury and repair										
MCP	73	124	110	156	73	71	59	89	**p < 0.001**	**p < 0.001**
Angio 1	72	13203	5930	20999	67	4138	1308	8299	**p < 0.001**	P = 0.14
Angio 2	72	3293	2421	4382	67	5744	4586	7970	**p < 0.001**	**p < 0.001**
Ang 2:Ang 1	72	0.27	0.16	0.46	67	1.51	0.67	3.5	**p < 0.001**	**p < 0.001**
E-selectin	73	13.7	10.2	21.5	73	20.7	12.9	26.1	**p = 0.002**	**p < 0.001**
P-selectin	73	59.0	49.0	82.6	73	45.0	37.3	61.4	**p < 0.001**	**p = 0.001**
sICAM	73	1070	728	1863	73	1069	715	1653	p = 0.57	**p < 0.001**
VCAM	73	1408	954	2516	73	1184	765	1796	**p = 0.04**	**p < 0.001**
VEGF	73	216	65	413	73	43	22.7	78.5	**p < 0.001**	**p = 0.001**
Disordered thrombogenesis										
D-dimer	73	27.7	5.9	196	73	5.9	5.9	53.6	**p = 0.01**	**p < 0.001**
TF	73	44.2	38.0	58.6	71	39.8	27.7	46.9	**p = 0.001**	**p = 0.003**
TM	73	4.2	3.4	5.2	73	4.5	3.9	5.1	**p = 0.02**	**p < 0.001**

** using Mann-Whitney U test; IL-1Ra, interleukin-1 receptor antagonist; CRP, C reactive protein; TNFa, tumour necrosis factor A; IL-10, interleukin-10; IL-6, interleukin-6; IL-8, interleukin-8; ICAM-3, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; Angio 1, angiopoietin-1; Angio 2, angiopoietin-2, Ang 2: Ang 1, ratio of angiopoietin 2:1; sICAM, soluble intracellular adhesion molecule; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; TF, tissue factor; TM, thrombomodulin.*

Significantly higher in ART-naïve vs controls, ART-experienced vs controls and ART-naïve vs experienced
Significantly higher in ART-naïve vs controls and vs ART-experienced; no evidence of difference between ART-experienced vs controls
Significantly (or marginally) higher in ART-naïve vs controls and vs ART-experienced; significantly lower in ART-experienced vs controls
Significantly lower in ART-naïve vs controls and in ART-experienced vs controls; significantly higher in ART-naïve vs experienced

Note: patterns seen in >1 biomarker indicated by shading

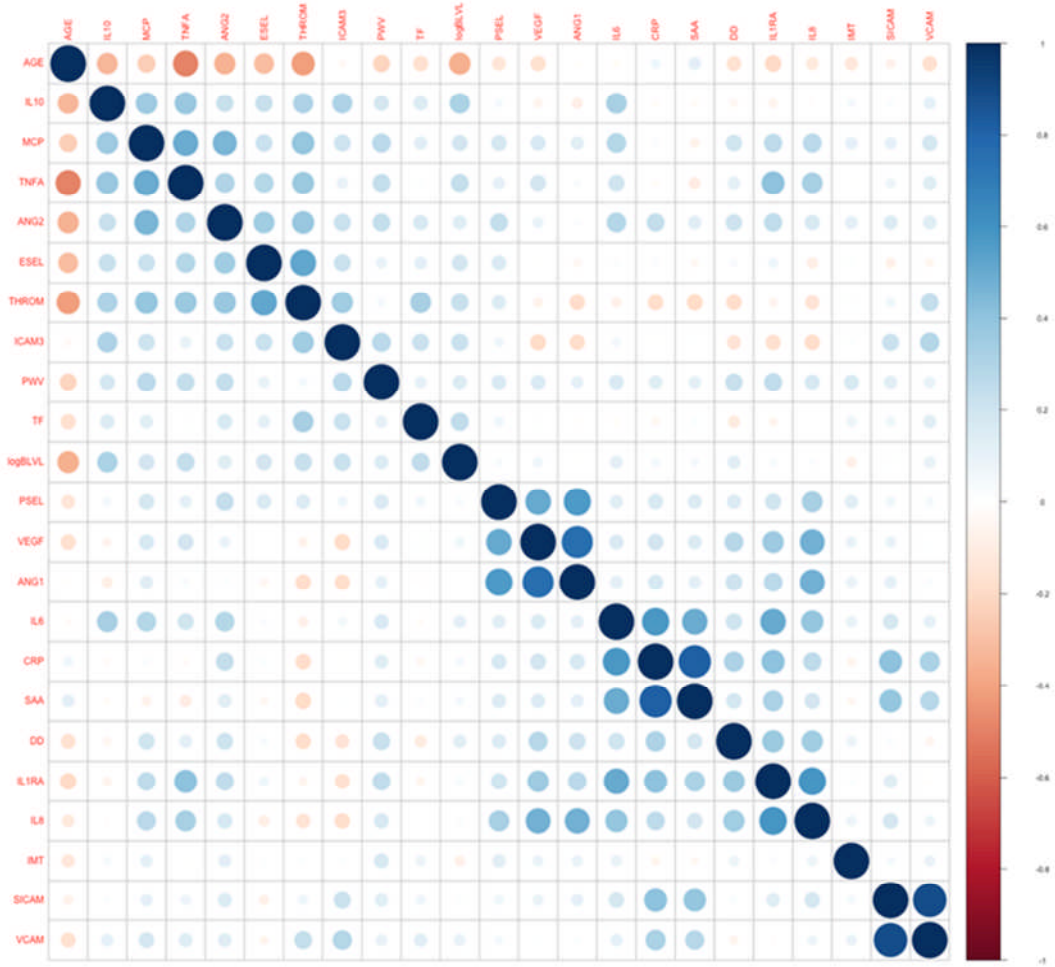
6.5 Relationship between the different biomarkers.

Associations between different biomarkers, viral load, IMT and PWV were investigated in each group (ART naïve, ART experienced and HIV uninfected) at baseline using univariable Spearman correlation co-efficients.

ART naive

As illustrated in Figure 6-7A, baseline IL-10, MCP-1, TNFa, Angiopoeitin-2, E-selectin, thrombomodulin and viral load were all significantly higher in the younger children within the ART-naïve group, with TNFa, thrombomodulin and viral load each having a correlation coefficient $r>0.35$ (Figure 6-7B). In this group of children, age is equivalent to duration of untreated HIV infection. The only factor associated with baseline viral load with $r>0.35$ was age. All correlations between IMT, PWV and the biomarkers were $r<0.35$ in magnitude. However, the other biomarkers demonstrated groupings that did not completely reflect what might have been predicted. There were strong positive correlations between:

- CRP, IL-6 and serum amyloid A
- VCAM and soluble ICAM; which were more closely related to CRP and serum amyloid A than E-selectin and P-selectin, the other markers of endothelial injury
- VEGF, P-selectin, angiopoietin-1 and IL-8; which were not closely related to angiopoietin-2
- IL-8 and IL-1Ra; IL-1Ra in particular was the common link between the CRP/IL-6/ serum amyloid A / sICAM / VCAM cluster above, the IL-8 / VEGF / angiopoietin-1 / P-selectin cluster above, and a cluster containing slightly more weakly linked factors including age, HIV viral load, E-selectin, TNFa, thrombomodulin, IL-10, MCP-1 and angiopoietin-2.


AGE
IL10
MCP
TNFA
ANG2
ESEL
THROM
ICAM3
PWV
TF
logBLVL
PSEL
VEGF
ANG1
IL6
CRP
SAA
DD
IL1RA
IL8
IMT
SICAM
VCAM

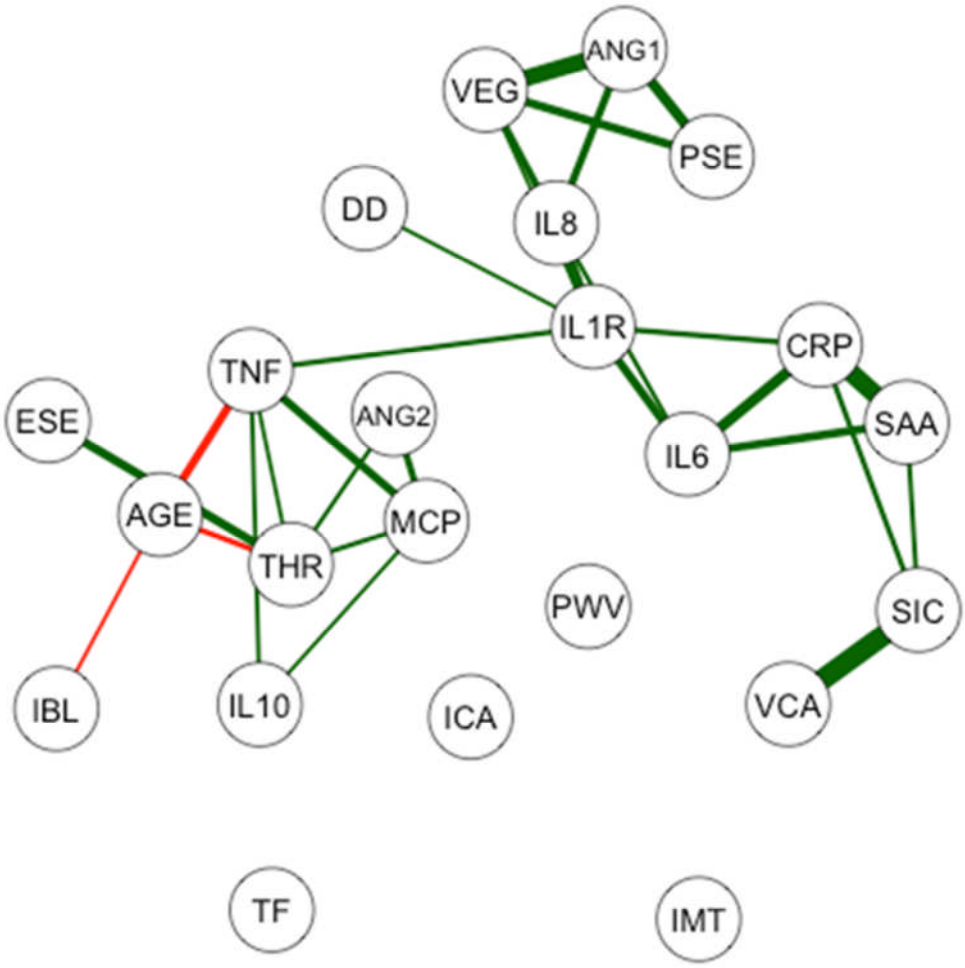


Figure 6-7. Relationship between biomarkers, viral load, age, IMT and PWV at baseline in ART naïve HIV-infected children.

In the correlation matrix (A) the strength of positive relationships are demonstrated using blue scale and negative correlations on the red scale. In the nodal diagram (B) relationships with a spearman correlation of >0.35 are demonstrated, positive correlations shown in green and negative correlations in red.

IL1R, interleukin-1 receptor antagonist; CRP, C reactive protein; TNF, tumour necrosis factor A; IL10, interleukin-10; IL6, interleukin-6; IL8, interleukin-8; ICA, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; ANG1, angiopoietin-1; ANG2, angiopoietin-2; SIC, soluble intracellular adhesion molecule; VCA, soluble vascular cell adhesion molecule-1; VEG, vascular endothelial growth factor; TF, tissue factor; THR, thrombomodulin; ESE, E-selectin; IBL, baseline viral load; DD, d-dimer; PSE, P-selectin; IMT, intimal medial thickness; PWV, pulse wave velocity.

ART experienced

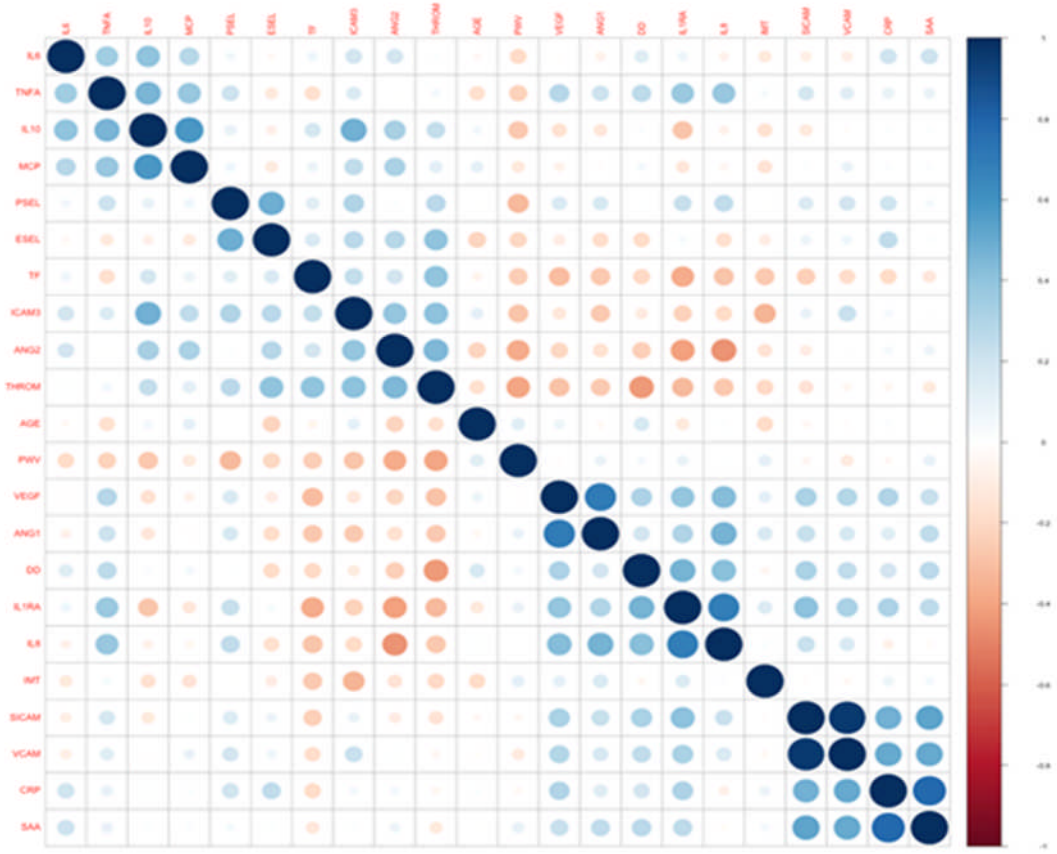
As illustrated in Figure 6-8A, age was much less strongly associated with biomarkers in HIV-infected, ART experienced children (all correlations <0.35), who had been on ART for at least two years as well as being older than the ART naïve children (medium age 2.9 versus 6.9 years). No significant correlations were seen between IMT and any biomarker, however PWV was higher in children with lower angiopoietin-2 and ICAM-3 levels. Several groups of biomarkers were significantly positively correlated with each other, some similarly to patterns observed in naïve children and others in contrast:

Similarly to naïve children

- Serum amyloid A, sICAM, CRP and VCAM
- TNFa, IL-10 and MCP-1
- IL-8, VEGF and angiopoietin-1
- IL-1Ra was highly associated with IL-8, and was also the common node linking the three clusters above

In contrast to naïve children

- P-selectin was highly associated with E-selectin (rather than VEGF / angiopoietin-1 in naïve children); E-selectin was still most associated with thrombomodulin
- IL-6 was now most strongly associated with IL-10, with little association with, IL-1Ra, IL-8. P-selectin, E-selectin, tissue factor, thrombomodulin, VEGF or angiopoietin-1.
- A more complex set of relationships existed between angiopoietin-2 and other factors including PWV


IL6
TNFA
IL10
MCP
PSEL
ESEL
TF
ICAM3
ANG2
THROM
AGE
PWV
VEGF
ANG1
DD
IL1RA
IL8
IMT
SICAM
VCAM
CRP
SAA

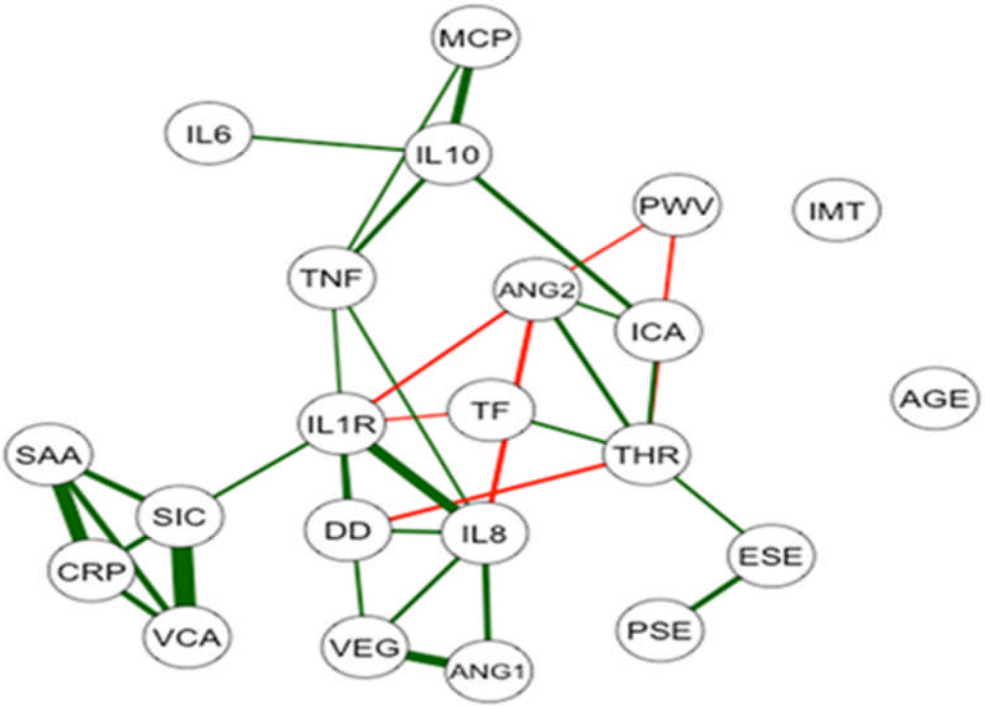


Figure 6-8. Relationship between biomarkers, viral load, age, IMT and PWV at baseline in ART experienced HIV-infected children.

In the correlation matrix (A) the strength of positive relationships are demonstrated using blue scale and negative correlations on the red scale. In the nodal diagram (B) relationships with a spearman correlation of >0.35 are demonstrated, positive correlations shown in green and negative correlations in red.

IL1R, interleukin-1 receptor antagonist; CRP, C reactive protein; TNF, tumour necrosis factor A; IL10, interleukin-10; IL6, interleukin-6; IL8, interleukin-8; ICA, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; ANG1, angiopoietin-1; ANG2, angiopoietin-2; SIC, soluble intracellular adhesion molecule; VCA, soluble vascular cell adhesion molecule-1; VEG, vascular endothelial growth factor; TF, tissue factor; THR, thrombomodulin; ESE, E-selectin; IBL, baseline viral load; DD, d-dimer; PSE, P-selectin; IMT, intimal medial thickness; PWV, pulse wave velocity.

HIV uninfected controls

As illustrated in Figure 6-9, older uninfected control children had significantly lower values of angiopoietin-2 and TNFa. IMT had only a weak positive correlation with thrombomodulin and PWV a weak positive correlation with P-selectin and E-selectin.

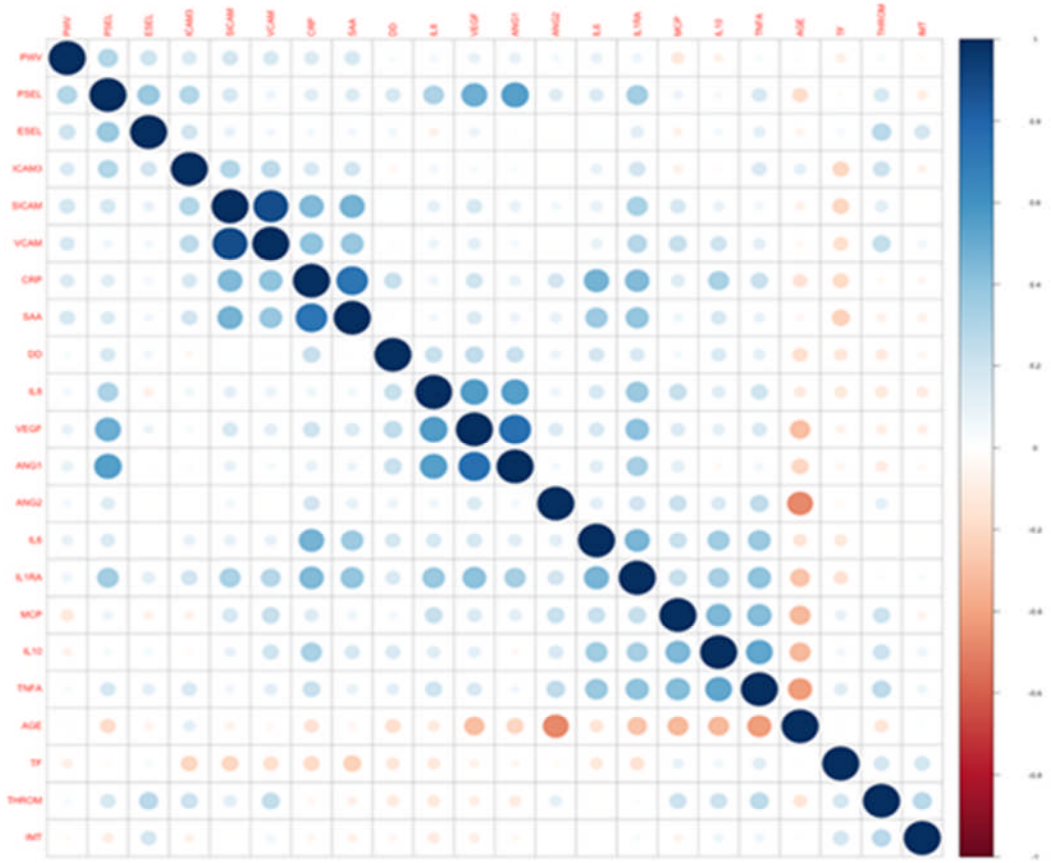
Interestingly, the same sets of biomarkers were significantly positively correlated with each other as observed in both naïve and experienced HIV-infected children:

- Serum amyloid A, soluble ICAM, CRP and VCAM
- IL-8, VEGF, angiopoeitin-1
- TNFa, IL-10 and MCP-1
- IL-1Ra was also the common node linking the three clusters above

Other strong associations reflected a mixture of those seen in ART-naïve or experienced children

- IL-6 was strongly associated with CRP, serum amyloid A and IL1-Ra, as seen in naïve but not experienced children
- E-selectin was strongly associated with P-selectin, as seen in experienced but not naïve children

In contrast to both groups of HIV-infected children, thrombomodulin was not strongly associated with any other biomarker in naïve children.


PWV
PSEL
ESEL
ICAM1
SICAM
VCAM
CRP
SAA
DD
IL8
VEGF
ANG1
ANG2
IL6
IL1RA
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IL10
TNFA
AGE
TF
THROM
IMT

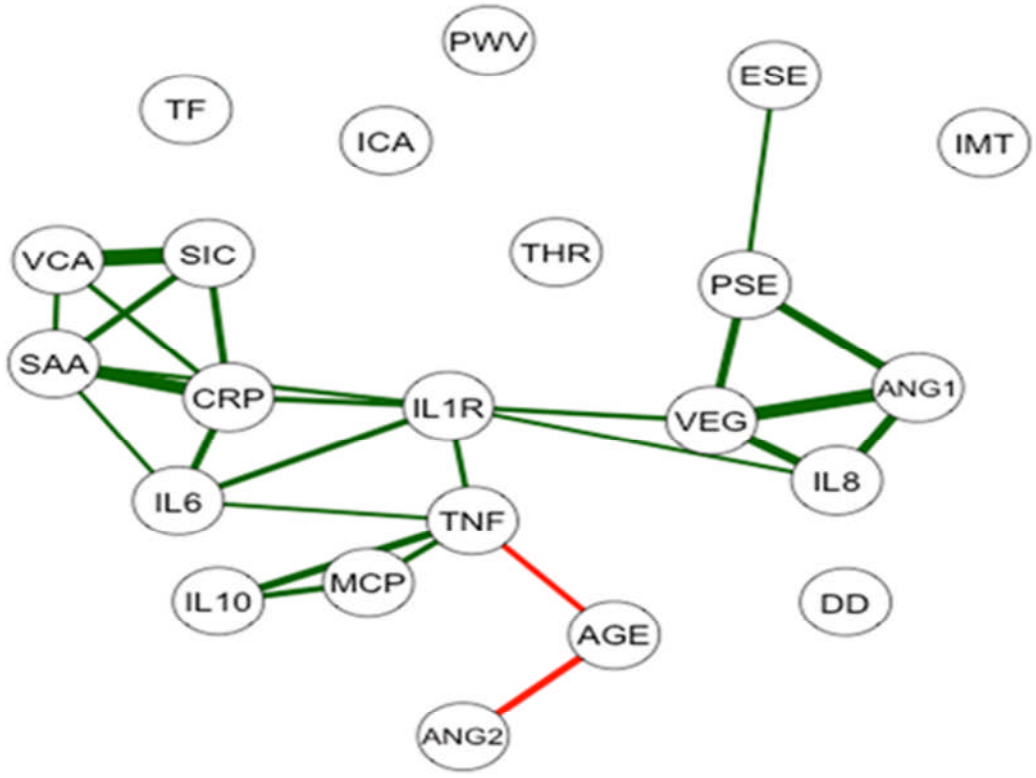


Figure 6-9. Relationship between biomarkers, viral load, age, IMT and PWV at baseline in HIV uninfected children.

In the correlation matrix (A) the strength of positive relationships are demonstrated using blue scale and negative correlations on the red scale. In the nodal diagram (B) relationships with a spearman correlation of >0.35 are demonstrated, positive correlations shown in green and negative correlations in red.

IL1R, interleukin-1 receptor antagonist; CRP, C reactive protein; TNF, tumour necrosis factor A; IL10, interleukin-10; IL6, interleukin-6; IL8, interleukin-8; ICA, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; ANG1, angiopoietin-1; ANG2, angiopoietin-2; SIC, soluble intracellular adhesion molecule; VCA, soluble vascular cell adhesion molecule-1; VEG, vascular endothelial growth factor; TF, tissue factor; THR, thrombomodulin; ESE, E-selectin; IBL, baseline viral load; DD, d-dimer; PSE, P-selectin; IMT, intimal medial thickness; PWV, pulse wave velocity.

6.6 Effect of age in HIV uninfected children

As little data on normal levels of these biomarkers in HIV-uninfected children are available I investigated correlations in more detail. Looking at the biomarker levels by age in the HIV uninfected controls, only certain patterns emerged. For 7/8 inflammatory biomarkers a significant negative correlation with increasing age existed (p all≤0.01). No significant difference by age was seen for serum amyloid A levels (p=0.35), Figure 6-10.

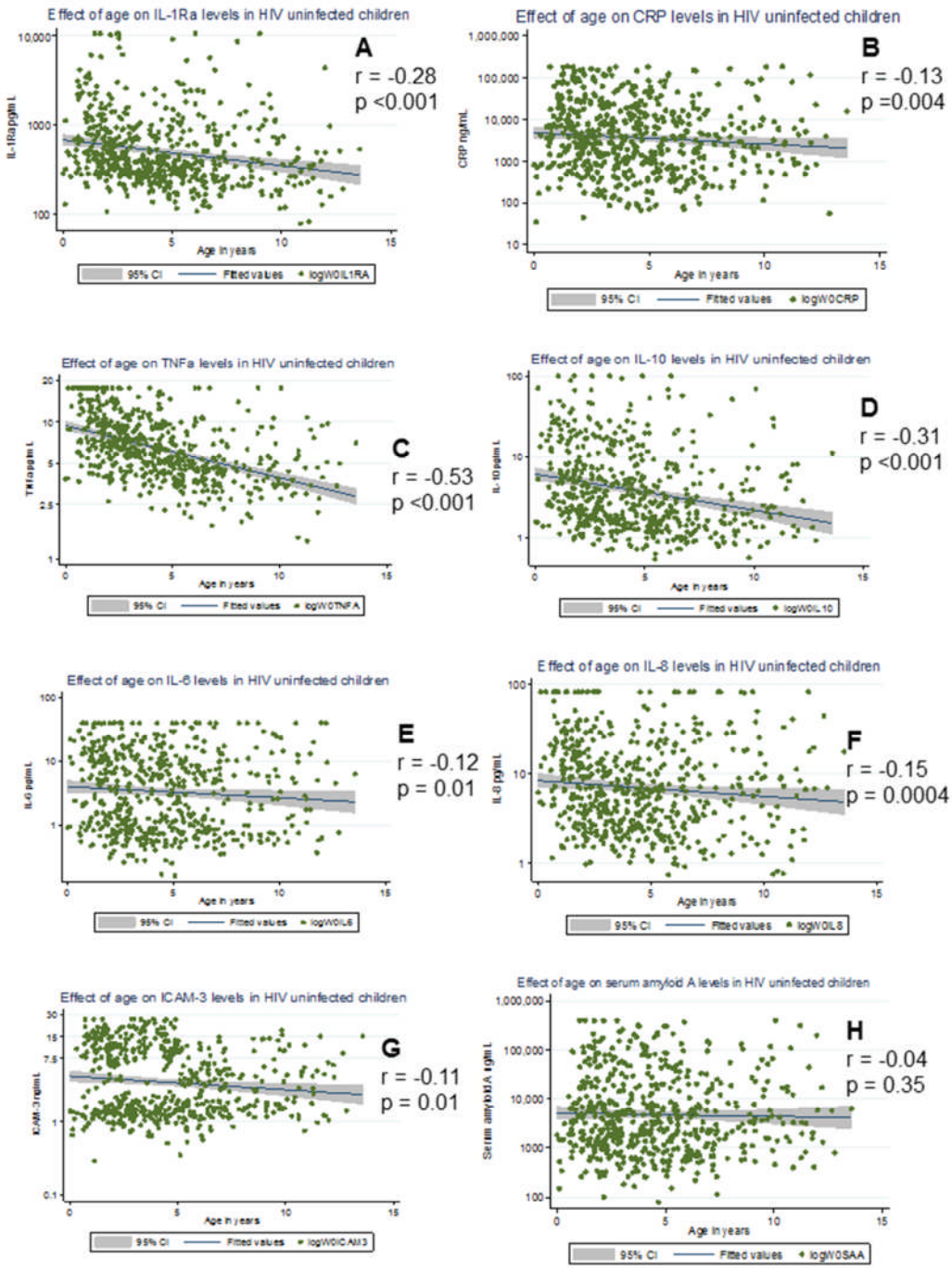


Figure 6-10. Effect of age on inflammatory biomarker level in HIV uninfected children.

With the exception of serum amyloid A a significant negative correlation with lower values observed in older children

A similar pattern was observed for 8/9 markers of cardiovascular injury and repair with a significant negative correlation with lower values observed in older children ($p \leq 0.04$). No significant difference by age was seen for angiopoietin-1 levels ($p=0.11$), Figure 6-11.

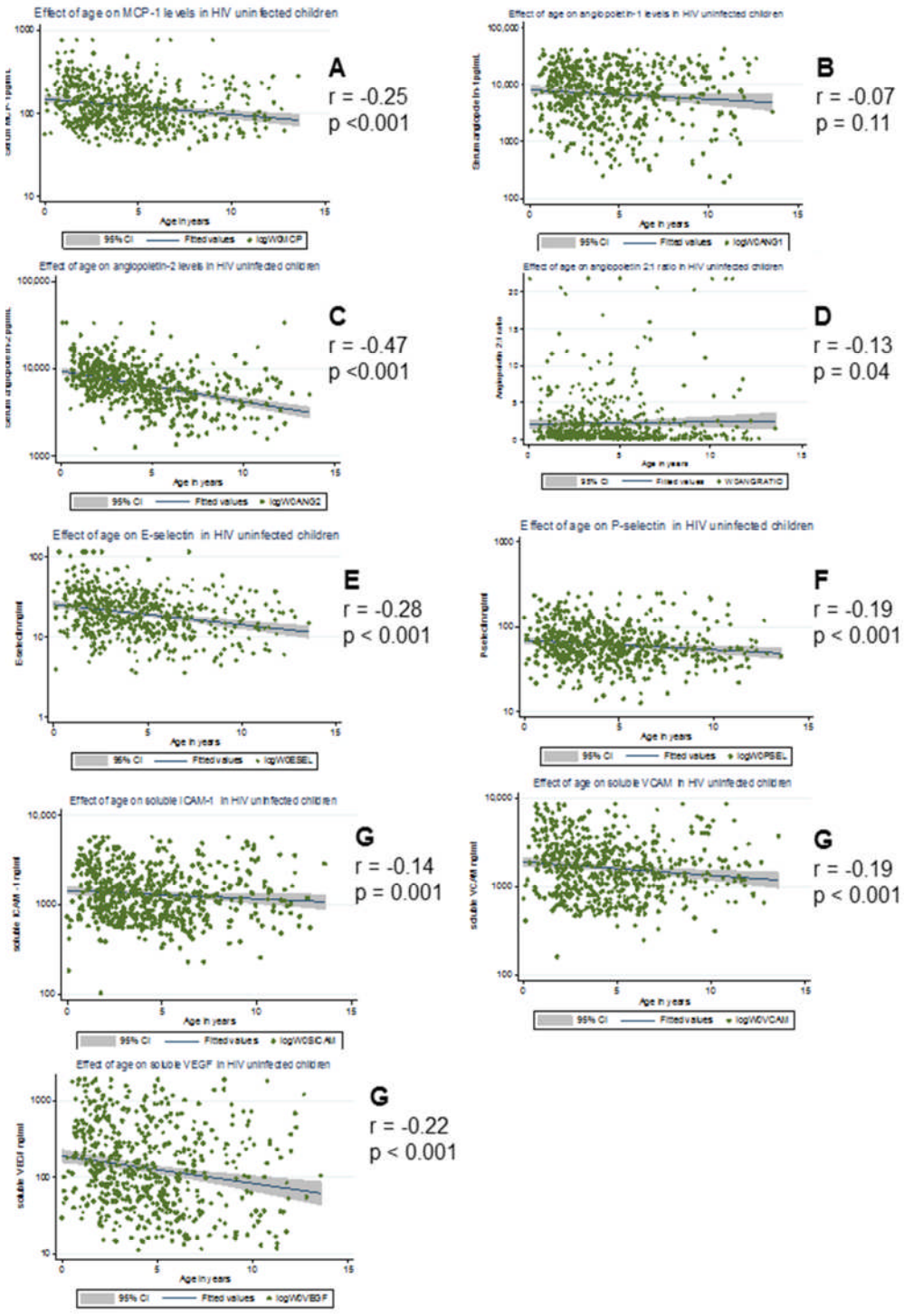


Figure 6-11. Effect of age on biomarkers of cardiovascular injury and repair in HIV uninfected children.

With the exception of angiopoeitin-1 all biomarker levels have a significant negative correlation with increasing age.

Looking at markers of disordered thrombogenesis, D-dimer and thrombomodulin levels decreased significantly with increasing age (p<0.001). No significant effect of age on tissue factor levels was seen (Figure 6-12).

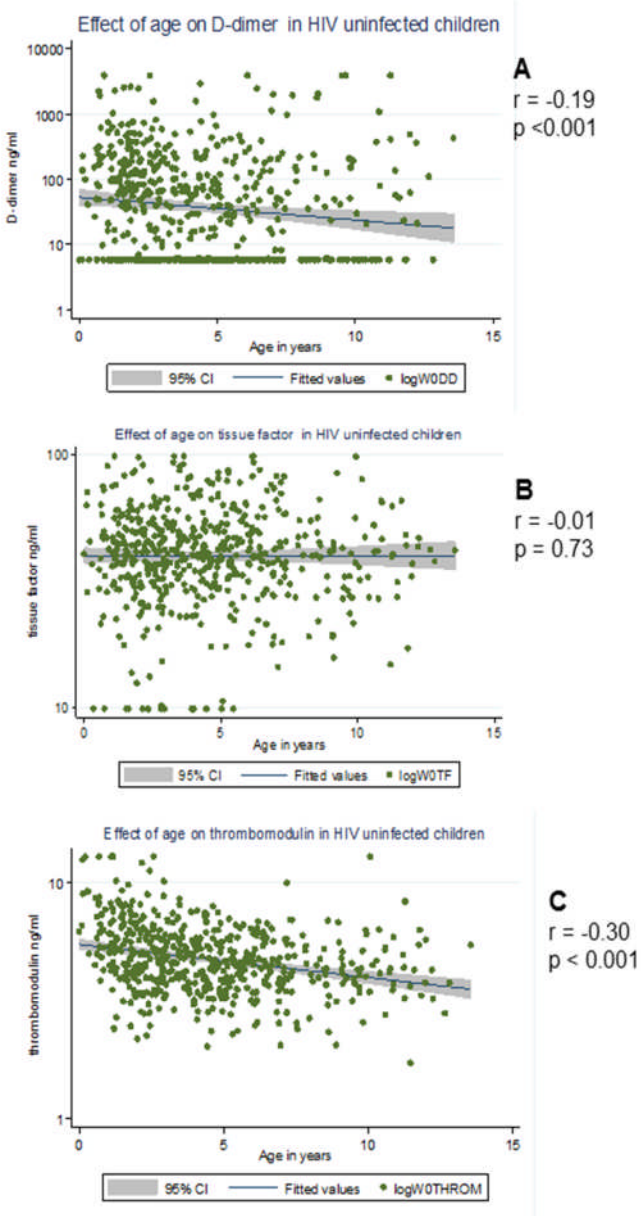


Figure 6-12. Effect of age on biomarkers of disordered thrombogenesis in HIV uninfected children.

D-dimer and thrombomodulin levels were significantly negatively corerlated with increasing age. No significant relationship between tissue factor levels and age were demonstrated.

6.7 Effect of age on biomarkers in HIV infected v HIV uninfected children

The above models simply look at pairwise correlations separately in each group of children. However, just as for IMT and PWV in Chapter 5, I can consider separately the ART-naïve children and their controls, and the ART-experienced children and their controls. First, I investigated the associations between age and biomarker values within each case-control group similarly to Chapter 5. In each case the biomarker is the dependent variable (outcome), and age and case-control group are independent variables (exposures). From the analyses above, in naïve children I would expect to find negative associations between age and TNFa, thrombomodulin and angiopoetin-2, with potentially different effects in cases vs controls for the latter two. Additional associations with age may also become evident due to greater power from combining cases and controls. In the smaller group of ART-experienced children and controls, I may find no associations due to lower power to detect effects, or possibly associations previously evident in the larger control group overall with TNFa and angiopoetin-2.

The results of the multivariate models are presented in Table 6-5. Within the smaller ART experienced group and their controls no significant effects of age or interactions dependent upon HIV status were found. In the larger ART-naïve group, in addition to the expected significant effect of age on TNFa levels, significant associations between age and levels of IL-1Ra, IL-10, MCP-1, VCAM, VEGF and d-dimer were demonstrated. Additionally the levels of angiopoietin-2, thrombomodulin, CRP, IL-6 and E-selectin were significantly associated with age, with differing effects of age in cases and controls.

Table 6-5. Results of multivariate analyses using a main effects and interaction model to screen for the effects of HIV status and age on each of the baseline biomarkers.

	ART naïve v controls		ART experienced v controls	
	p value for main effect of age adjusted for group	p value for interaction by age and HIV status	p value for main effect of age adjusted for group	p value for interaction by age and HIV status
Inflammatory				
IL-1Ra	**0.01**	0.10	0.06	0.65
CRP	NA	**0.02**	0.61	0.51
TNFa	**<0.001**	0.70	0.37	0.14
IL-10	**<0.001**	0.57	0.46	0.36
IL-6	NA	**0.03**	0.57	0.97
IL-8	0.70	0.23	0.53	0.72
ICAM-3	0.96	0.42	0.13	0.98
SAA	0.48	0.07	0.73	0.86
Cardiovascular injury and repair				
MCP-1	**<0.001**	0.16	0.78	0.71
Angio 1	0.39	0.46	0.23	0.67
Angio 2	NA	**0.03**	0.38	0.96
Ang 2 : Ang 1	0.23	0.67	0.08	0.43
E-selectin	NA	**0.02**	0.13	0.44
P-selectin	0.10	0.62	0.44	0.34
sICAM	0.42	0.24	0.28	0.31
VCAM	**0.04**	0.75	0.07	0.14
VEGF	**0.002**	0.55	0.56	0.37
Disordered thrombogenesis				
TM	NA	**0.02**	0.99	0.15
D-DIMER	**0.004**	0.09	0.17	0.61
TF	0.37	0.34	0.82	0.66

IL-1Ra, interleukin-1 receptor antagonist; CRP, C reactive protein; TNFa, tumour necrosis factor A; IL-10, interleukin-10; IL-6, interleukin-6; IL-8, interleukin-8; ICAM-3, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; Angio 1, angiopoietin-1; Angio 2, angiopoietin-2, Ang 2: Ang 1, ratio of angiopoietin 2:1; sICAM, soluble intracellular adhesion molecule; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; TF, tissue factor; TM, thrombomodulin.

Expected associations from the univariable analaysis

Table 6-6 shows the multivariate models for the seven biomarkers which were associated with age but with no evidence that this age association varied by case-control status. All biomarkers decreased as age at ART initiation increased (inverse association), and were independently significantly lower in controls than cases.

Table 6-6. Results of a linear regression without interaction terms to look at effect of age and HIV status on baseline biomarker results.

Biomarker and factor	Co-efficient	95% CI	p value
IL-1Ra			
Baseline age (increase per year)	-0.019	(-0.034 : -0.004)	**0.01**
ART naïve v control	-0.206	(-0.278 : -0.135)	**<0.001**
Constant	2.914	(2.842 : 2.986)	**<0.001**
TNFa			
Baseline age (increase per year)	-0.029	(-0.035 : -0.023)	**<0.001**
ART naïve v control	-0.234	(-0.264 : -0.204)	**<0.001**
Constant	1.082	(1.052 : 1.112)	**<0.001**
IL-10			
Baseline age (increase per year)	-0.047	(-0.061 : -0.033)	**<0.001**
ART naïve v control	-0.654	(-0.723 : -0.586)	**<0.001**
Constant	1.136	(1.067 : 1.204)	**<0.001**
MCP-1			
Baseline age (increase per year)	-0.014	(-0.021 : -0.008)	**<0.001**
ART naïve v control	-0.380	(-0.412 : -0.346)	**<0.001**
Constant	2.357	(2.324 : 2.390)	**<0.001**
VCAM			
Baseline age (increase per year)	-0.011	(-0.022 : -0.001)	**0.04**
ART naïve v control	-0.355	(-0.407 : -0.304)	**<0.001**
Constant	3.459	(3.407 : 3.511)	**<0.001**
VEGF			
Baseline age (increase per year)	-0.026	(-0.042 : -0.010)	**0.002**
ART naïve v control	-0.680	(-0.758 : -0.603)	**<0.001**
Constant	2.602	(2.524 : 2.680)	**<0.001**
d-dimer			
Baseline age (increase per year)	-0.040	(-0.067 : -0.013)	**0.004**
ART naïve v control	-0.671	(-0.802 : -0.539)	**<0.001**
Constant	2.094	(1.961 : 2.226)	**<0.001**

IL-1Ra, interleukin-1 receptor antagonist; TNFa, tumour necrosis factor A; IL-10, interleukin-10; MCP-1, Monocyte chemoattractant protein-1; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor.

Table 6-7 shows the multivariate models for the five biomarkers which were associated with age and with evidence that this age association varied by case-control status. For 3 biomarkers (angiopoietin-2, E-selectin and thrombomodulin) levels significantly decreased as age at ART initiation increased in HIV infected patients (p≤0.03). In controls similar significant decreased levels were seen with increasing age for IL-6, angiopoietin-2 and thrombomodulin (p≤0.02).

Table 6-7. Results of a linear regression with interaction terms to look at effect of age and HIV status on baseline biomarker results

Biomarker and factor	Co-efficient	95% CI	p value
CRP			
Increase per year older in HIV infected	0.023	(-0.015 : 0.068)	0.20
Increase per year older in controls	-0.044	(-0.090 : 0.017)	0.06
IL-6			
Increase per year older in HIV infected	0.008	(-0.011 : 0.026)	0.41
Increase per year older in controls	-0.022	(-0.043 : -0.002)	**0.03**
Angio-2			
Increase per year older in HIV infected	-0.018	(-0.039 : -0.007)	**0.002**
Increase per year older in controls	-0.036	(-0.050 : -0.023)	**<0.001**
E-selectin			
Increase per year older in HIV infected	-0.034	(-0.047 : -0.020)	**<0.001**
Increase per year older in controls	-0.009	(-0.024 : 0.007)	0.26
Thrombomodulin			
Increase per year older in HIV infected	-0.023	(-0.031 : -0.016)	**<0.001**
Increase per year older in controls	-0.010	(-0.019 : -0.002)	**0.02**

CRP, C reactive protein; IL-6, interleukin-6; Angio 2, angiopoietin-2.

6.8 The influence of baseline viral load and age on the biomarkers in HIV infected, ART naïve children?

To investigate the impact of baseline viral load and age on biomarker levels a bivariable analysis was performed on children in group 1 (HIV+ ART naïve) only, since these were the only children within this study with any variation in VL. As shown in Table 6-8 older children had significantly lower levels of TNFa ($p<0.001$) and serum amyloid A ($p=0.02$) with no significant effect of age on these biomarkers. A higher baseline viral load was significantly associated with higher baseline levels of IL-6 ($p=0.04$), ICAM-3 ($p<0.001$), MCP-1 ($p=0.03$) and tissue factor ($p=0.04$) with no evidence of an independent effect of age on these biomarkers. In particular, the significant effects of age on MCP-1 shown in Table 6-6 are therefore due to confounding between VL and age, with VL being the more important predictor of these biomarkers. Both age and baseline viral load significantly influenced levels of IL-10 ($p\leq0.01$), E-selectin ($p\leq0.02$) and thrombomodulin ($p\leq0.04$).

Table 6-8. Influence of age and baseline viral load on baseline biomarkers in HIV infected, ART naive children.

	Age			log baseline VL		
	co-efficient	95%CI	p value	co-efficient	95%CI	p value
Inflammatory						
IL-1Ra	-0.002	(-0.028 : 0.024)	0.88	0.044	(-0.046 : 0.133)	0.34
CRP	0.041	(-0.002 : 0.084)	0.06	0.123	(-0.027 : 0.272)	0.11
TNFa	-0.026	(-0.036 : -0.017)	**<0.001**	0.033	(0.000 : 0.067)	0.05
IL-10	-0.042	(-0.065 : -0.018)	**0.001**	0.106	(0.024 : 0.188)	**0.01**
IL-6	0.014	(-0.005 : 0.034)	0.14	0.070	(0.004 : 0.137)	**0.04**
IL-8	0.012	(-0.016 : 0.040)	0.40	0.071	(-0.027 : 0.169)	0.15
ICAM-3	0.003	(-0.009 : 0.015)	0.62	0.082	(0.040 : 0.124)	**<0.001**
SAA	0.053	(0.008 : 0.098)	**0.02**	0.111	(-0.046 : 0.268)	0.16
Cardiovascular injury and repair						
MCP-1	-0.006	(-0.017 : 0.006)	0.32	0.043	(0.004 : 0.083)	**0.03**
Angio 1	-0.002	(-0.031 : 0.027)	0.89	0.027	(-0.073 : 0.127)	0.59
Angio 2	-0.013	(-0.027 : 0.000)	0.05	0.033	(-0.014 : 0.079)	0.17
Ang 2: Ang 1	-0.097	(-0.289 : 0.096)	0.32	0.066	(-0.602 : 0.733)	0.85
E-selectin	-0.027	(-0.043 : -0.011)	**0.001**	0.064	(0.009 : 0.119)	**0.02**
P-Selectin	-0.005	(-0.019 : 0.008)	0.43	0.026	(-0.020 : 0.072)	0.26
sICAM	0.000	(-0.016 : 0.016)	0.99	0.015	(-0.039 : 0.070)	0.59
VCAM	-0.010	(-0.026 : 0.006)	0.20	0.028	(-0.026 : 0.083)	0.30
VEGF	-0.021	(-0.048 : 0.005)	0.11	0.026	(-0.065 : 0.117)	0.58
Disordered thrombogenesis						
D-dimer	-0.005	(-0.046 : 0.036)	0.81	0.131	(-0.012 : 0.274)	0.07
TF	-0.004	(-0.012 : 0.004)	0.30	0.028	(0.001 : 0.055)	**0.04**
TM	-0.021	(-0.030 : -0.012)	**<0.001**	0.033	(0.002 : 0.064)	**0.04**

IL-1Ra, interleukin-1 receptor antagonist; CRP, C reactive protein; TNFa, tumour necrosis factor A; IL-10, interleukin-10; IL-6, interleukin-6; IL-8, interleukin-8; ICAM-3, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; Angio 1, angiopoietin-1; Angio 2, angiopoietin-2, Ang 2: Ang 1, ratio of angiopoietin 2:1; sICAM, soluble intracellular adhesion molecule; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; TF, tissue factor; TM, thrombomodulin.

6.9 Is there a relationship between angiopoietin 1/2 and VEGF?

Angiopoietin 1 and 2 and VEGF are involved in remodelling and endothelial cell migration; an imbalance in the relationships between these three biomarkers associated with HIV infection may reflect ongoing vascular injury. As illustrated in Figure 6-13 a significant relationship exists between the ratio of angiopoietin 2:1 and VEGF levels. Additionally a trend by group also appears with the majority of HIV infected ART naïve children having higher VEGF and Angiopoietin 2:1 ratios whilst HIV infected, ART experienced children have generally lower VEGF and angiopoietin 2:1 ratios.

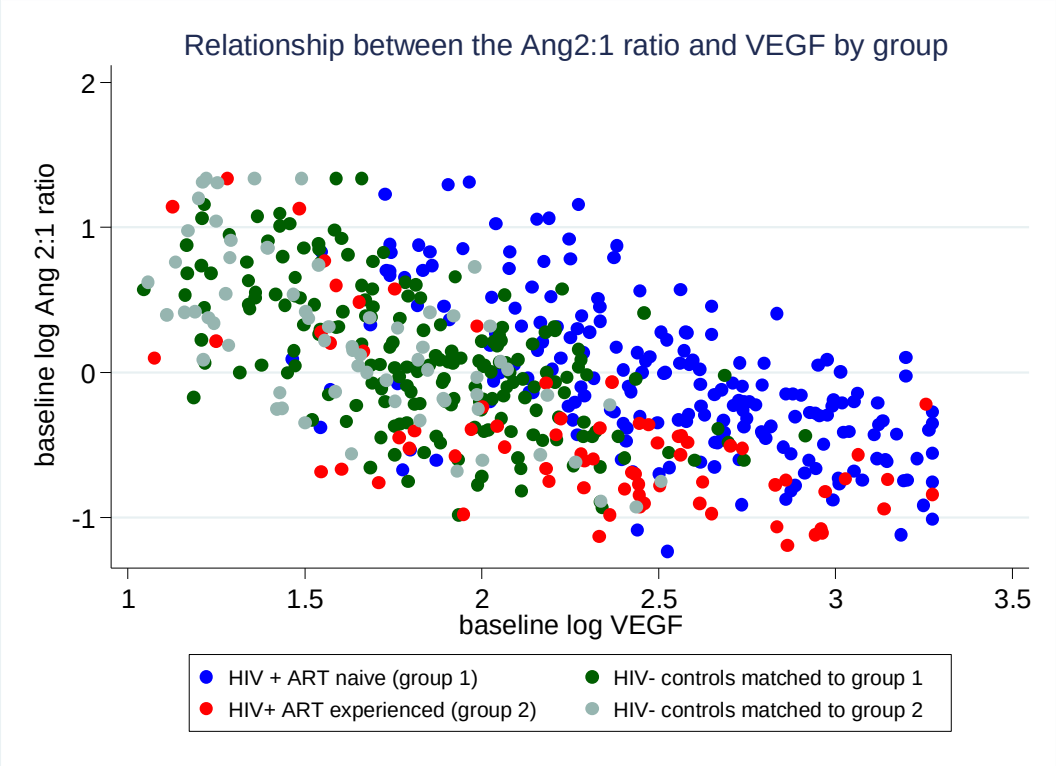


Figure 6-13. Relationship between the ratio of angiopoietin 2 to 1 with VEGF by group.

A significant relationship between VEGF and the angiopoietin ratio was demonstrated, in that for every 1 log higher VEGF, the angiopoietin ratio was (-)0.609 log lower (95%CI -0.680 ; -0.538), p<0.001). In an interaction model, there was no evidence that the relationship between the VEGF and angiopoietin ratio differed by group (HIV+ART naïve, HIV+ ART experienced or their controls), p≥0.13.

To determine whether the effect of age confounds the relationship between VEGF and the angiopoietin ratio, I included age as an independent factor in this model. First there was no evidence that the impact of age on the log angiopoetin 2:1 ratio varied according to case control status, but there was strong evidence that, overall, the log angiopoetin 2:1 ratio was (-)0.029 lower for every year older (95% CI -0.043 to -0.016) p<0.001). In the HIV-infected, ART naive and HIV-infected, ART experienced groups, the association between age and log angiopoetin 2:1ratio was similar (heterogeneity p=0.76), with log angiopoetin 2:1 ratio being (-)0.757 and (-)0.723 lower for every year older, respectively. However, overall log angiopoetin 2:1ratio levels were significantly lower in HIV-infected, ART experienced group, by (-)0.530 log (p=0.04). In contrast, overall levels were similar in HIV-infected, ART naive and control groups (p=0.99), but log angiopoetin 2:1 ratio declined more quickly with older age in control group, by (-)0.944 log for every year older (heterogeneity p=0.046).

To determine whether the relationship is further modified by the baseline viral load this was added to the model but was not significant (p=0.87).

6.10 Longitudinal changes in biomarkers.

The effects over 96 weeks of commencing ART or continuing ART were looked at. In naïve children 16/19 biomarkers at week 96 were significantly lower than the baseline levels ($p \leq 0.001$). The remaining 3 biomarkers had no significant change from baseline. In experienced children 12/19 biomarkers fell significantly over 96 weeks ($p \leq 0.02$) whilst 3/19 had significant increases and 4/19 had no significant differences over 96 weeks. The changes over 96 weeks in each biomarker are summarised in Table 6-9 and illustrated in Figures 6-14 to 6-17.

Table 6-9. Changes in biomarkers over 96 weeks by group.

		Week 0			Week 48			Week 96			Change over 96 weeks		
	Group	No	Median	IQR	No	Median	IQR	No	Median	IQR	Median	log10 difference	p value
Inflammatory													
IL-1Ra	1	205	586	357 1150	185	314	247 515	174	262	181 418	-213	-0.32	**<0.001**
	2	73	289	178 652	73	271	184 396	74	209	168 372	-57	-0.12	**0.01**
CRP	1	205	8675	28223 3441	185	5829	1620 23736	174	2919	1120 13053	-2333	-0.31	**<0.001**
	2	73	3791	1283 10781	73	1945	827 11517	74	1162	414 3939	-1097	-0.38	**<0.001**
TNFa	1	205	9.6	7.1 13	185	5.7	4.7 7	174	4.4	3.4 5.8	-4.8	-0.32	**<0.001**
	2	73	3.7	3.1 5.1	73	3.8	3 5	74	3.1	2.8 4	-0.6	-0.08	**0.01**
IL-10	1	205	7.8	4.7 18	185	2.4	1.5 4.1	174	1.3	0.9 2.5	-5.8	-0.72	**<0.001**
	2	73	2.7	1.8 7.2	73	2.1	1.6 4.1	74	1	0.7 1.5	-1.4	-0.4	**<0.001**
IL-6	1	205	11.9	7 20.8	185	1.6	0.8 6.7	174	0.9	0.6 1.5	-9.9	-1.09	**<0.001**
	2	73	6.4	4.6 10.3	73	5.2	2.2 9.7	74	0.7	0.5 1.1	-5.7	-1.02	**<0.001**
IL-8	1	205	12.1	5.7 27.3	185	4.6	2.9 9.6	174	4.7	2.6 9.2	-5.6	-0.35	**<0.001**
	2	73	6.8	3.1 11.6	73	5.9	2.5 10.3	74	3.8	2.5 5.9	-1.2	-0.13	**0.02**
ICAM-3	1	204	11.9	8.6 16.1	184	2	1.3 5.3	174	1.1	0.8 1.4	-11	-1.03	**<0.001**
	2	73	4.4	3.4 5.3	73	3.7	2.3 5.2	74	1	0.8 1.2	-3.4	-0.65	**<0.001**
SAA	1	205	5583	1592 27357	185	4225	1418 23962	174	2370	949 8719	-810	-0.19	**<0.001**
	2	73	2368	1207 11843	73	2616	1161 7310	74	1324	529 3631	-618	-0.25	**0.01**
Cardiovasular injury and repair													
MCP-1	1	205	202.6	155 267.1	185	95.2	71.4 128.4	174	63.8	52.8 81.3	-123	-0.48	**<0.001**
	2	73	124.2	109.6 155.6	73	114.2	88.1 136.4	74	55	46.5 64.6	-68	-0.35	**<0.001**
Angio 1	1	197	10579	4011 17581	181	6137	3199 14112	167	8691	3299 1785	371	0.05	0.91
	2	72	13203	5930 20999	73	10375	4725 18240	71	9279	4164 14897	-2291	-0.08	0.08
Angio 2	1	197	6485	5120 9039	181	6127	4232 9141	167	6337	4838 9369	461	0.03	0.96
	2	72	3293	2421 4382	73	3532	2292 4984	71	5726	4467 7127	2290	0.25	**<0.001**
Ang 2: Ang 1	1	197	-0.14	-0.41 0.27	181	-0.007	-0.38 0.33	167	-0.08	-0.43 0.3	-0.07	-0.05	0.89
	2	72	-0.57	-0.79 -0.33	73	-0.47	-0.66 -0.15	71	-0.14	-0.46 0.18	0.24	0.37	**<0.001**
E-selectin	1	204	25.3	16.1 38.2	184	18.5	12.8 32.2	174	16.1	11.5 26.3	-8.6	-0.19	**<0.001**
	2	73	13.7	10.2 21.5	73	18.4	12.1 26.5	74	18.3	11.3 28.5	3.3	0.1	**0.002**
P-Selectin	1	204	78	54 115	184	46	35 61	174	55	39 91	-14	-0.13	**<0.001**
	2	73	59.1	49.1 82.6	73	46.4	36 58.2	74	67	51 95	8.3	0.05	0.16
sICAM	1	205	1613	1113 2603	185	980	680 1298	174	628	504 873	-1011	-0.45	**<0.001**
	2	73	1071	728 1863	73	869	647 1152	74	542	451 659	-511	-0.29	**<0.001**
VCAM	1	205	2549	1820 4009	185	1016	726 1542	174	632	490 877	-1901	-0.63	**<0.001**
	2	73	1408	954 2516	73	1038	700 1445	74	498	450 630	-863	-0.46	**<0.001**
VEGF	1	205	3564	158 725	185	94	45 169	174	63.3	29 126	-215	-0.67	**<0.001**
	2	73	216	65 413	73	120	44 274	74	45	25 78	-158	-0.6	**<0.001**
Disordered thrombogensis													
D-dimer	1	204	101	37 247	184	39	6.6 144	174	20	4.4 117	-41	-0.58	**<0.001**
	2	73	28	5.9 196	73	37	5.5 128	74	10.3	6.6 76	-5.7	-0.31	0.09
TF	1	203	41	34 50	184	42	33 53	174	46	37 59	4.9	0.04	0.06
	2	73	44	38 59	73	41	35 51	74	44	34 61	-1.6	-0.02	0.16
TM	1	204	5	3.9 6.4	184	3.9	3.1 5	174	4.2	3.2 5.4	-0.5	-0.05	**<0.001**
	2	73	4.2	3.4 5.2	73	3.7	3 4.5	74	4.8	3.8 5.6	0.9	0.09	**0.004**

Paired t-test on log10 values used to calculate changes over time.

Significant reduction in week 96 levels compared to baseline

Significant increase in week 96 levels compared to baseline

Note: patterns seen in >1 biomarker indicated by shading

Inflammatory markers (IL-1Ra, CRP, TNFa, IL-10, IL-6, IL-8, ICAM-3, SAA)

Significant decreases in the levels of all 8 markers of inflammation were demonstrated over the 96 weeks in both ART naïve and ART experienced children - Figure 6-14.

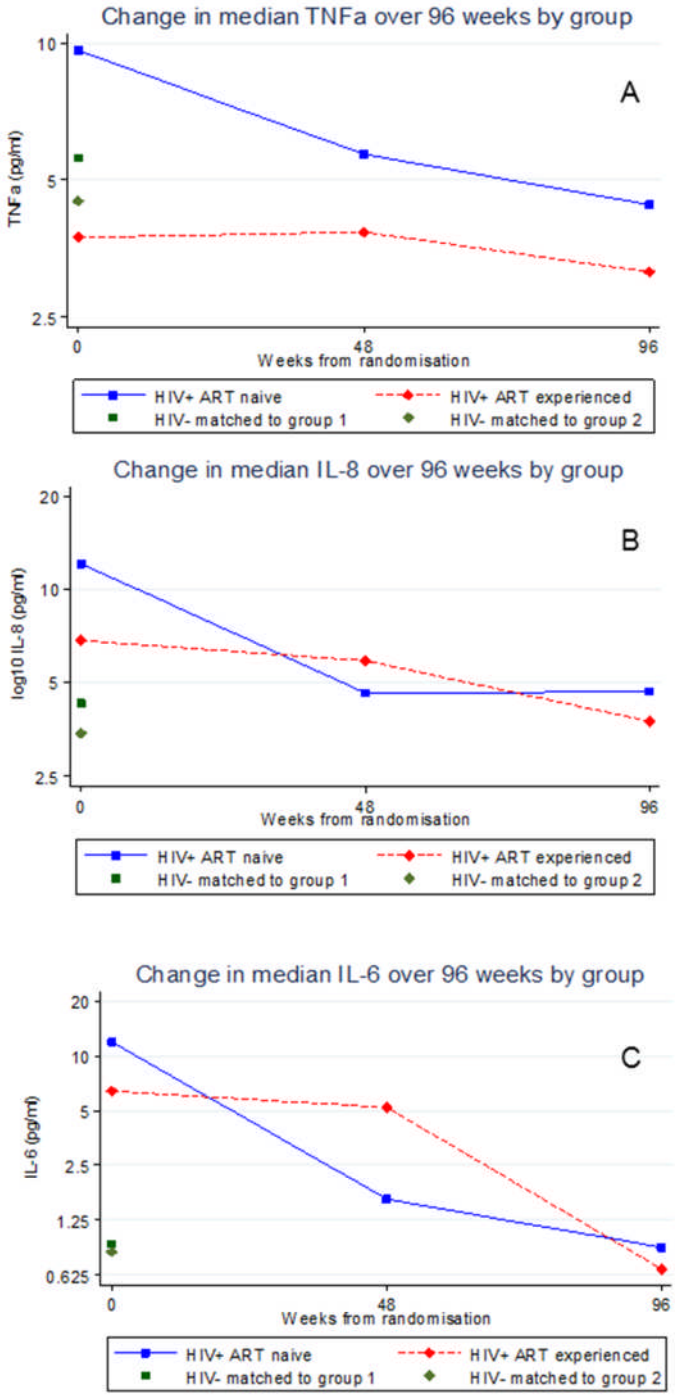


Figure 6-14. Examples of changes over 96 weeks in 3 of the inflammatory biomarker panel studied by baseline ART exposure.

Levels seen in HIV uninfected children shown at baseline where they are matched for age to each infected groups. A) TNFa, B) IL-8, C) IL-6

Markers of cardiovascular injury and repair (MCP-1, Ang-1, Ang-2, E-selectin, P-selectin, sICAM-1, VCAM, VEGF)

Significant decreases were seen in the levels of MCP-1, VEGF, sICAM, and VCAM in both ART naïve and ART experienced children over 96 weeks to levels lower than those of HIV uninfected controls. E-selectin levels fell significantly in in ART naïve children and increased significantly in ART experienced children over 96 weeks. Conversely P-selectin levels fell significantly over 96 weeks in ART naïve children whilst no significant difference was seen in ART experienced children between week 96 and baseline - Figure 6-15.

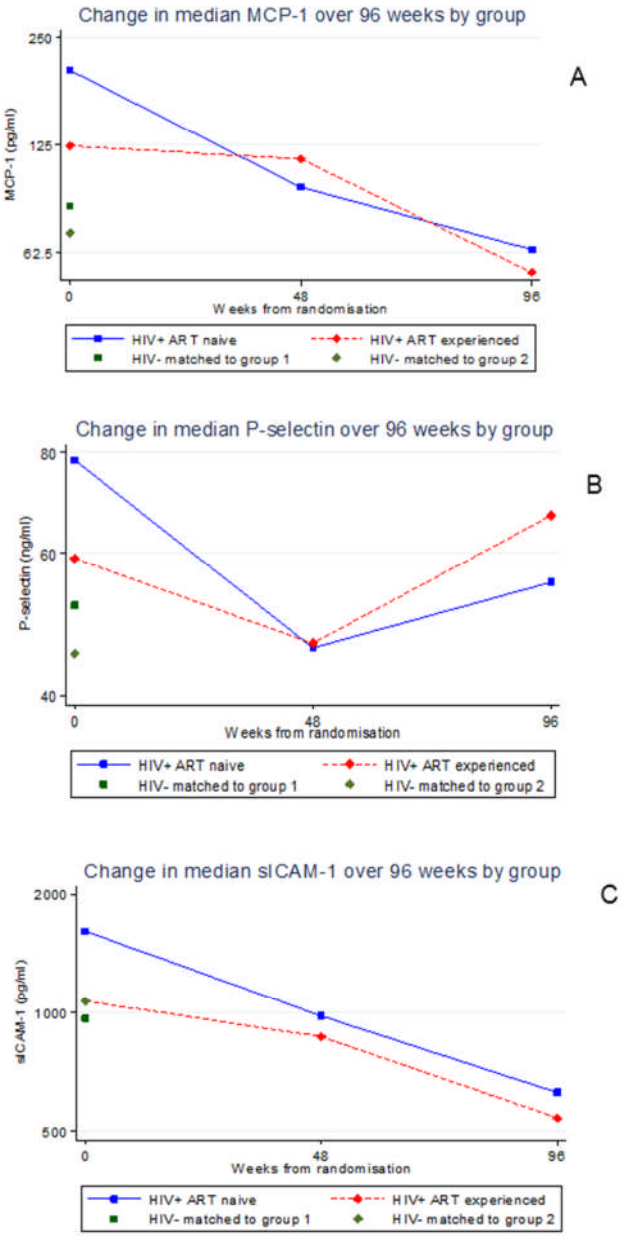


Figure 6-15. Examples of changes over 96 weeks in 3 of the biomarkers reflecting cardiovascular injury and repair by baseline ART exposure.

Angiopoietin 1 levels did not change significantly over 96 weeks in either ART naïve or ART experienced children. Angiopoietin 2 levels did significantly increase over 96 weeks in ART experienced children, but did not change significantly in ART-naïve children. As expected therefore, the Angiopoietin 2:1 ratio did not significantly change in ART naïve children over 96 weeks however a significant increase was seen in ART experienced children - Figure 6-16

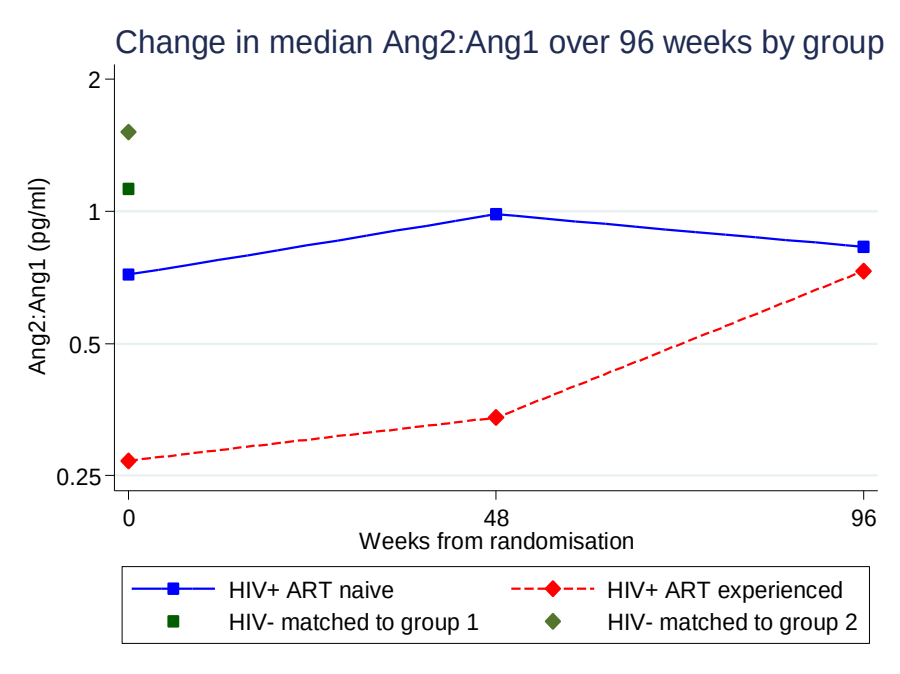


Figure 6-16. Changes in the ratio of Angiopoietin 2 to angiopoietin 1 over 96 weeks by ART exposure at baseline.

HIV uninfected control results shown at baseline.

Markers of disordered thrombogenesis (d-dimer, TF, TM)

A small but significant increase in tissue factor levels over 96 weeks was seen in the ART naïve group, no significant differences were seen over time in the ART experienced group. A significant decrease in ART naïve children and a significant increase in ART experienced children in thrombomodulin was observed. D-dimer levels significantly fell in both ART naïve and experienced groups - Figure 6-17.

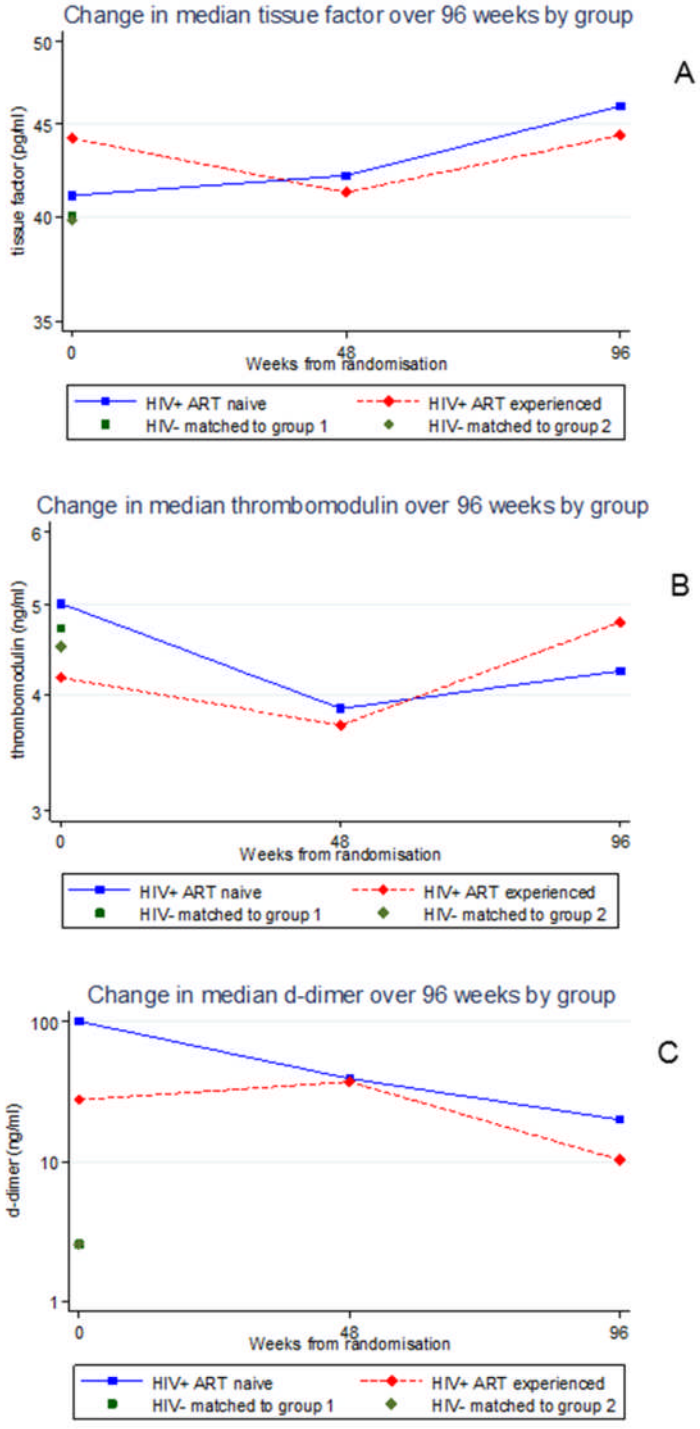


Figure 6-17. Examples of changes over 96 weeks in biomarkers reflecting disordered thrombogenesis by baseline ART exposure.

Levels seen in HIV uninfected children shown at baseline

6.11 Effect of randomized NRTI on change in biomarkers over 96 weeks

The Kruskal–Wallis non-parametric test was used to compare any differences across the three randomized NRTIs in the changes from baseline to week 96 in each of the log of the 19 biomarkers, separately in ART-naïve and ART-experienced strata (total 36 tests). Where the Kruskal-Wallis test had p<0.1, I then conducted pairwise tests between each pair of randomized NRTIs using the ranksum test. Two Kruskal Wallis p-values were <0.05 (change in CRP from baseline in ART-naive p=0.047 and change in IL-10 from baseline in ART-experienced p=0.02). There were a further 3 results where the p value was between 0.05-0.1 suggesting a trend towards a difference by NRTI - Table 6-10. If there were no differences between randomized NRTI groups, I would expect 0.05*36=1.8 tests to have p<0.05, and 0.1*36=3.6 tests to have p<0.1 by chance; therefore these results likely reflect multiple testing rather than genuine differences.

Table 6-10. Biomarkers with borderline significant results suggesting effect of arm affecting change from baseline

	Group	Kruskal Wallis	Mann Whitney		
			d4T v ZDV	ZDV v ABC	d4T v ABC
IL-1Ra	2	p = 0.07	p = 0.12	p = 0.37	**p = 0.03**
CRP	1	**p = 0.05**	p = 0.27	p = 0.13	**p = 0.02**
IL-10	2	**p = 0.02**	p = 0.003	p = 0.19	p = 0.24
IL-8	2	p = 0.08	p = 0.86	p = 0.08	**p = 0.03**
SAA	1	p = 0.06	p = 0.19	p = 0.23	**p = 0.02**

6.12 The impact of viral load suppression on the change in biomarkers

To determine the influence of week 96 virological suppression separate models were fitted for both ART naïve and ART experienced **-** Table 6-11 and Table 6-12**.**

Table 6-11. Results of multivariate models investigating the effect of age, baseline viral load and virological suppression on change in each biomarker over 96 weeks in children who were ART naïve at baseline.

Each model also adjusted for log10 baseline biomarker (to adjust for possible regression to the mean).

NAIVE	Age		Baseline VL		Virological suppression at wk 96	
	Co-efficient	p	Co-efficient	p	Co-efficient	p
Inflammatory						
IL1-Ra	0.01	0.87	0.05	0.22	-0.23	**<0.001**
CRP	-0.02	0.41	0.06	0.47	-0.26	0.05
TNFa	0.04	0.32	0.02	0.37	-0.19	**<0.001**
IL-10	-0.04	**0.001**	0.03	0.50	-0.13	0.05
IL-6	-0.01	0.71	0.06	0.24	-0.23	**0.01**
IL-8	0.01	0.46	0.02	0.78	-0.20	**0.02**
ICAM-3	0.01	0.36	0	0.98	-0.22	**<0.001**
SAA	-0.03	0.29	0.15	**0.08**	-0.20	0.14
Cardiovascular injury and repair						
MCP-1	-0.01	**0.05**	0.02	0.31	-0.12	**<0.001**
Angio 1	-0.01	**0.05**	0.02	0.31	-0.04	0.58
Angio 2	-0.01	0.20	0.03	0.20	-0.13	**<0.001**
E-selectin	0.00	0.95	-0.05	**0.10**	-0.11	**0.02**
P-Selectin	0.00	0.72	-0.04	0.14	-0.08	0.08
sICAM	-0.01	0.16	0.02	0.40	-0.13	**<0.001**
VCAM	-0.01	**0.05**	0.02	0.35	-0.15	**<0.001**
VEGF	-0.03	**0.01**	-0.02	0.68	-0.13	**0.03**
Disordered thrombogenesis						
D-dimer	0.01	0.81	0.04	0.76	-0.53	**0.01**
TF	-0.01	0.21	0.01	0.58	0.12	**<0.001**
TM	0.00	0.46	-0.02	0.19	0.00	0.19

VL, viral load; IL-1Ra, interleukin-1 receptor antagonist; CRP, C reactive protein; TNFa, tumour necrosis factor A; IL-10, interleukin-10; IL-6, interleukin-6; IL-8, interleukin-8; ICAM-3, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; Angio 1, angiopoietin-1; Angio 2, angiopoietin-2; sICAM, soluble intracellular adhesion molecule; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; TF, tissue factor; TM, thrombomodulin.

Table 6-12. Results of multivariate models investigating the effect of age and virological suppression on change in each biomarker over 96 weeks in children who were ART experienced at baseline.

Model adjusted for log10 baseline biomarker values to adjust for potential regression to the mean.

EXPERIENCED	Age		Virological suppression at wk 96	
	Co-efficient	p	Co-efficient	p
Inflammatory				
IL1-Ra	0.01	0.71	-0.25	0.09
CRP	0.08	0.07	-1.10	**0.001**
TNFa	0.00	0.89	-0.12	0.14
IL-10	0.03	0.29	-0.39	0.06
IL-6	0.07	**0.002**	-0.30	0.12
IL-8	-0.01	0.55	-0.38	**0.04**
ICAM-3	0.01	0.38	0.00	0.99
SAA	0.12	**0.01**	-1.32	**0.001**
Cardiovascular injury and repair				
MCP-1	0.02	0.12	-0.07	0.38
Angio 1	-0.02	0.55	-0.29	0.18
Angio 2	0.01	0.08	-0.10	0.10
E-selectin	0.01	0.57	-0.17	0.17
P-Selectin	0.01	0.54	-0.04	0.70
sICAM	0.02	0.06	-0.24	**0.01**
VCAM	0.12	**0.04**	-0.18	**0.02**
VEGF	0.02	0.33	-0.39	**0.02**
Disordered thrombogenesis				
D-dimer	-0.02	0.63	-0.46	0.33
TF	-0.01	0.42	0.01	0.84
TM	0.01	0.15	-0.13	0.07

VL, viral load; IL-1Ra, interleukin-1 receptor antagonist; CRP, C reactive protein; TNFa, tumour necrosis factor A; IL-10, interleukin-10; IL-6, interleukin-6; IL-8, interleukin-8; ICAM-3, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; Angio 1, angiopoietin-1; Angio 2, angiopoietin-2; sICAM, soluble intracellular adhesion molecule; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; TF, tissue factor; TM, thrombomodulin.

For each biomarker, a model that then included age, baseline viral load, whether ART naïve or experienced at trial entry and week 96 virological suppression was then fitted in all children. As shown in Table 6-13 week 96 suppression was the fundamental factor significantly affecting change in 16/19 of the biomarkers, in all cases with virological suppression associated with larger decreases from baseline. There was no significant effect of virological suppression at week 96 on change in angiopoietin-1 (p=0.39), whilst for both P-selectin and thrombomodulin there was marginal evidence of an association in the same direction as for other biomarkers (p=0.08). Having adjusted for virological suppression at week 96, the only biomarkers whose change remained associated with age were ICAM-3 and IL-10. Older children had smaller decreases in ICAM-3, but larger decreases in IL-10. There were no additional effects of children being ART-naïve vs experienced at enrolment or of baseline VL.

Table 6-13. Results of a multivariable regression model to look at the effect of baseline age, ART naïve/experienced, baseline viral load and virological suppression at week 96.

Model adjusted for log10 biomarker results.

	Age at baseline		Effect of ART naïve / experienced at baseline		Baseline log viral load		Viral load suppression at week 96	
	Co-efficient	p	Co-efficient	p	Co-efficient	p	Co-efficient	p
Inflammatory								
IL1-Ra	0.010	0.27	0.220	0.41	0.050	0.20	-0.240	**<0.001**
CRP	0.003	0.90	-0.040	0.90	0.070	0.38	-0.350	**0.003**
TNFa	0.040	0.20	0.020	0.83	0.020	0.26	-0.190	**<0.001**
IL-10	-0.020	**0.05**	0.130	0.40	0.030	0.56	-0.180	**0.01**
IL-6	0.011	0.35	0.120	0.35	0.080	0.12	-0.240	**0.002**
IL-8	0.007	0.62	-0.070	0.69	0.009	0.86	-0.220	**0.004**
ICAM-3	0.160	**0.01**	0.020	0.80	-0.001	0.97	-0.190	**<0.001**
SAA	0.008	0.72	0.440	0.72	0.170	0.05	-0.330	**0.01**
Cardiovascular injury and repair								
MCP-1	-0.006	0.30	0.090	0.21	0.030	0.19	-0.120	**<0.001**
Angio 1	-0.008	0.52	0.110	0.53	0.030	0.57	-0.060	0.39
Angio 2	-0.003	0.62	0.200	0.62	0.030	0.10	-0.120	**<0.001**
E-selectin	0.002	0.84	-0.080	0.44	-0.050	0.08	-0.110	**0.01**
P-Selectin	0.005	0.53	-0.060	0.57	-0.040	0.13	-0.070	0.08
sICAM	-0.003	0.63	0.080	0.29	0.020	0.27	-0.130	**<0.001**
VCAM	-0.005	0.37	0.070	0.34	0.030	0.20	-0.150	**<0.001**
VEGF	-0.020	0.07	0.050	0.70	-0.010	0.81	-0.160	**0.01**
Disordered thrombogenesis								
D-dimer	-0.001	0.94	0.300	0.45	0.030	0.78	-0.520	**0.002**
TF	-0.007	0.15	-0.001	0.99	0.007	0.70	0.110	**<0.001**
TM	0.060	0.17	-0.020	0.74	-0.020	0.16	-0.040	0.08

VL, viral load; IL-1Ra, interleukin-1 receptor antagonist; CRP, C reactive protein; TNFa, tumour necrosis factor A; IL-10, interleukin-10; IL-6, interleukin-6; IL-8, interleukin-8; ICAM-3, intracellular adhesion molecule-3; SAA, soluble amyloid A; MCP, Monocyte chemoattractant protein-1; Angio 1, angiopoietin-1; Angio 2, angiopoietin-2; sICAM, soluble intracellular adhesion molecule; VCAM, soluble vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; TF, tissue factor; TM, thrombomodulin.

6.13 Discussion

The biomarker panels were put together a priori based upon existing literature focusing upon the way people classically thought about pathogenesis in terms of markers of inflammation, vascular damage / repair and disordered thrombogenesis but the results presented within this chapter have shown some interesting relationships and many markers span more than one group. This study represents, to my knowledge, the first systematic and prospective longitudinal study of biomarkers in HIV-infected African children, which is matched to cardiovascular and immunological markers. Children received a limited repertoire of ART combinations, the majority of children were virologically supressed at week 96 and the population studied allowed the effects of HIV and ART to be looked at without confounders such as a diverse ethnic population, co-morbidities multiple ART combinations and obesity influencing levels [437, 438].

Results from other paediatric HIV studies are summarised in Table 6-14 and whilst interesting, the populations studied are diverse in terms of geographical location, ART history and several include horizontally infected adolescents. Studies were conducted in populations with a high incidence of severe HIV and ART related metabolic disturbances. The use of different biomarker panels, techniques and small sample sizes may explain some of the irreproducibility of results. Without exception all studies to date have studied older children / adolescents. As I have clearly demonstrated a clear inverse relationship with age and biomarkers exists for the majority of the biomarkers studied (except SAA, Ang-1 and tissue factor) so that all comparisons with the existing literature must consider the impact of age.

Table 6-14. Summary of studies of changes in biomarkers in HIV infected children

Number recruited	Setting	% patients on ART	Parameters measured	Findings	Ref
83 HIV + 59 HIV – Aged 5.4 – 17.7 years	UK.	33% ART naïve 67% on ART *(31/56 PI 25/56 non PI)*	cIMT, FMD, BP, anthropometry, total cholesterol, HDL, apoB, LPa, LDL, hsCRP	Lipid abnormality not associated with IMT / FMD. Increased hsCRP in HIV +	[241]
64 HIV + 30 HIV – mean age 14.1 years	Spain	All on ART, 83% with VL <50 copies/mL	IMT, IL-6, CRP. MPO. tPA, sVCAM-1, P-selectin, sCD14, lipid profile	HIV + higher sCD14, sVCAM. No relationship between biomarkers and VL. No effect HIV status for CRP, IL-6, MPO, MCP-1, P-selectin and tPA. No correlation between biomarkers and IMT.	[244]
77 HIV + 32 HV – 13% horizontally infected Median age 16.2 years	Spain	70% with undetectable VL 30% with VL > 50	hsCRP, D-dimer, β2-microglobulin, HLA-DR+CD38+, LPS, microbial 16srDNA, sCD14	HIV+ higher CRP, d-dimer, β2microglobulin; no ART class effect. HLA DR+CD38+ cells increased in viraemic children. Higher levels of LPS, 16s RNA and sCD14 independent of viraemia.	[439]
31 HIV+ stable on ART for >6 months 31 HIV -	USA	84% VL < 400 copies/ml 16/31on PI based ART	cIMT, homocysteine, hsCRP, myeloperoxidase, lipids	HIV+ higher cholesterol, triglycerides, MPO and lower homocysteine levels. BMI significant predictor of IMT in HIV-, duration of ART predictive of IMT in HIV+	[249]
106 HIV+, mean age 14.8 years, 5% horizontally infected 55 HIV -, mean age 12.3 years	USA	86% on ART	CRP, IL-6, MCP-1, fibrinogen, P-selectin, sICAM< sVCAM, E-selectin, leptin, anthropometry	HIV+; higher sICAM, sVCAM, MCP-1, IL-6, fibrinogen. Higher IL-6, MCP-1, CRP and sICAM correlated with higher waist:hip, sVCAM, MCP-1, IL-6, fibrinogen and CRP inversely related to CD4%.	[440]

Number recruited	Setting	% patients on ART	Parameters measured	Findings	Ref
226 HIV + median age 12.3 years 140 HEU median age 10.3 years	USA and Puerto Rico	89% on ART, 65% VL < 400 copies / mL	CRP IL-6 MCP-1 Fibrinogen P-selectin sICAM-1, sVCAM, E-selectin, adiponectin	HIV+ higher MCP-1, fibrinogen, sICAM and sVCAM. BMI z score associated with higher CRP and fibrinogen but lower MCP-1 and sVCAM. Higher VL associated with higher levels of MCP-1, CRP, sICAM and sVCAM.	[407]
88 HIV+, 10% horizontal transmission mean age 13.5 years	Italy	86% on ART, 72% VL < 1000 copies / mL	Protein S, protein C anticoagulant, antithrombin, fibrinogen, d-dimer, hsCRP and homocysteine.	High prevalence of thrombophilic abnormalities. Low protein S levels and high d-dimers associated with detectable VL. CRP unrelated to VL	[441]
27 HIV+ 30 HIV- mean age 11 years	USA	4% horizontally infected, 96% on ART, 70% VL < 50 copies / mL	IMT, fasting lipids, insulin, TNFα, sTNFR-I, II, IL-6, hsCRP, MPO, ICAM-1, sVCAM, VWF	No difference except for higher hsCRP in HIV+. CRP, age and female sex positively associated with increased IMT.	[442]
97 HIV + 81 HIV – horizontally infected adolescents	USA	67 on ART, 9% with VL suppression 30 ART naive	Lipids, hsCRP, sVCAM-1, myeloperoxidase and neopterin.	HIV+ on ART lower HDL and apoprotein A compared to HIV -, hsCRP not elevated and not predictive of risk. sVCAM-1 elevated in HIV+ in both ART naïve and experienced.	[409]
49 HIV+ aged 1 month to 13 years No controls	USA	29% ART naïve 22% on ART starting PI 49% switching ART due to treatment failure 2% VL < 400	VL, CD4, lipids, apo A and B, IGF-1, IGFBP-1, IGFBP-3, anthropometry, biochemical impedence, TNFA, IL-1B, IL-6	No drug class effect TNFα, levels fall with ART and related to CD4% – increase 10%CD4 count fall of 26% TNFα. Non-significant reduction in IL-6 with ART, inverse relationship between IL-6 and CD4%	[443]
100 HIV + 50 HIV – Aged 12 – 20 years	Thailand	All on ART, 48% PI based, 82% undetectable VL	fasting lipid profiles, hs-CRP, NT-pro-BNP, echocardiogram, cIMT.	hsCRP no different from controls	[240]
sTNFR-I; soluble TNF receptors, MPO; myeloperoxidase					

6.13.1 Baseline results in HIV infected, ART naïve children

In HIV infected, ART naïve 16 of the 19 biomarkers were higher than levels in age matched HIV uninfected controls. Angiopoeitin-2 levels were lower than seen in HIV uninfected controls and thrombomodulin and tissue factor levels were comparable in HIV infected and uninfected controls.

Compared to HIV infected children on ART 17/19 biomarkers were higher in the ART naïve children, Ang-1 levels were comparable regardless of ART status whilst tissue factor levels were lower in HIV infected children not on ART. However the ART experienced children were older than the ART naïve children.

Taken together these results add support to earlier initiation of ART, as advocated by current WHO guidelines, to reduce levels of inflammation and vascular injury.

6.13.2 The effects of ART and changes in biomarkers

At baseline ART experienced children, despite a median of 3.5 years of effective ART, had significant differences in 16/19 biomarkers compared to HIV uninfected controls. 12 (CRP, IL-10, IL-6, IL-8, ICAM-3, MCP-1, Ang-1, P-selectin, VCAM, VEGF, d-dimer and tissue factor) remained elevated compare to controls whilst TNFα, Ang-2, E-selectin and thrombomodulin levels were significantly lower than controls. IL-1Ra, serum amyloid A and sICAM levels were not significantly different between controls and cases.

Analysis of changes following 96 weeks of treatment revealed that in the children who were ART naïve and then commenced ART 16/19 biomarkers had significant decreases in levels; Ang-1, Ang-2 and tissue factor levels did not significantly change. In those children who were ART experienced at baseline 12/19 biomarkers continued to fall, 3 had significant increases in levels (Ang-2, E-selectin and thrombomodulin) whilst 4 had no significant change (Ang-1, P-selectin, D-dimer and tissue factor). This suggests that despite viral suppression further improvements are seen with ongoing ART. These findings add to literature showing that ART has been shown to decrease, although not completely normalise inflammatory biomarkers [77, 107, 444, 445]. The acknowledgement that ART does not or cannot normalise all of

these biomarkers may suggest that either children are not starting ART early enough or in spite of viral suppression, abnormalities remain.

Published paediatric results which suggest a role for active viral replication and elevated cardiovascular biomarkers have been published [407]. This is encouraging as with viral suppression pro-atherogenic mechanisms may be attenuated. Sainz and colleagues did not detect any significant association between biomarkers and VL [244]. Other studies have shown VL to be associated with markers of inflammation (MCP-1, CRP) and endothelial dysfunction (sICAM, sVCAM) [407].

6.13.3 What do biomarkers add to our understanding of paediatric HIV?

Extensive work in adults has examined the addition of multiple biomarkers to traditional risk prediction scores (such as the Framlington Risk Score) for cardiovascular disease. Whilst a number of circulating, genetic, and imaging biomarkers are robustly associated with cardiovascular risk the evidence that these biomarkers can improve individual cardiovascular risk prediction is limited [446, 447].

Pre-ART changes

Inflammation in early HIV disease progression is not well characterized. Ninety adults with untreated primary HIV-1 infection were studied to determine associations of inflammatory proteins with early disease progression. High TNF-α levels were significantly associated with an increased viral load set point and shorter times to ART initiation. The increased risk of reaching a CD4+ T-cell count of <500 cells/mm3 in the group with high TNF-α levels was driven by viral load but was independent of concurrent CD4+ T-cell count. Thus, TNF-α appears to be an important mediator of inflammation in patients with poor viral control and early HIV-1 disease progression [378]. Pre-ART inflammation and coagulation activation have not been shown to predict CD4 count response to ART in adults [448].

CRP

Elevated CRP has been shown in the general adult population to be consistently correlated with CVD, especially MI. CRP levels are positively correlated with BMI,

glycated haemoglobin levels, female sex and greater numbers of metabolic syndrome markers whilst negatively correlated with HDL cholesterol levels [449]. Several studies have reported a trend towards increased CRP in HIV infected children compared to HIV uninfected controls [241, 249].

Relationship between biomarkers and markers of cardiovascular structure and arterial stiffness

IMT and PWV are research tools that can provide insight into those patients who have increased cardiovascular risk. In order to perform both techniques equipment and training are required and they are both unlikely to be incorporated into routine clinical care. Measurement of a circulating biomarker as a surrogate marker is attractive however results to date are in keeping with other findings that no biomarker has been found that is predictive of cardiovascular changes [450, 451]. Studies of healthy children have reported an association of hsCRP and IMT [442]. In keeping with two other paediatric HIV studies [240, 244] we did not find an association between CRP and IMT. In an adult population soluble VCAM-1 was associated with higher CIMT independently of age and blood pressure in HIV-1 infected, virologically suppressed adults at risk for CVD [452], we were unable to demonstrate such an association.

Prediction of morbidity and mortality once on ART

The use of a biomarker to predict morbidity and mortality is attractive. Several studies have tried to do this for example after successful ART therapy d-dimer levels remain elevated and are significantly associated with HIV associated non AIDS events [453]. Higher IL-6 level and D-dimer levels at baseline and year 1 were associated with the occurrence of a non–AIDS-defining event [316]. The mortality and morbidity in CHAPS 3 was low and so the significance of elevated levels in these biomarkers in children could not be determined.

In conclusion I have shown that there are significant differences between biomarkers in HIV infected African children on and off ART compared to HIV uninfected children. For the first time in an African setting a large scale study of the

normal range of biomarker levels has been determined and this paves the way for future studies within an African setting.

Chapter 7 Results: Immunophenotyping

7.1 Introduction

7.1.1 Immune activation

In the early 1990s Giorgi et al showed that immune activation by HIV, as demonstrated by expression of CD38 on CD8 T-cells, was a very strong predictor of accelerated progression to AIDS [308]. Attention since has focused upon HLA DR+ and CD38+ expression on CD4+ and CD8+ T-cells as markers of activation in adults. Higher levels of activation are related to viral load, disease progression, increased morbidity and mortality from both AIDS and non AIDS related disease [94, 309-317, 454, 455]. ART reduces immune activation but higher levels of residual inflammation can remain despite a good virological response to ART; the greater the degree of immunosuppression at the time of commencing ART the higher the degree of residual inflammation and this can limit immune reconstitution. Less is known in HIV-infected children.

7.1.2 Thymic output

T-cells can be divided into naïve and memory subsets based upon the expression of CD45 isoforms. CD45 is a leukocyte common antigen of which 2 isoforms, RA and RO, exist. Naïve cells are characterized by CD45RA expression and are yet to 'see' antigen whilst CD45RO positive cells are usually memory cells. As introduced in section 3.9.1 early thymic output is crucial in establishing and maintaining the peripheral naïve T-cell pool in children [456]. In healthy children thymic output peaks at one year of age and then gradually decreases as increased peripheral T-cell division maintains the naive T-cell repertoire [456-458]. T-cells recently released from the thymus are CD31+, naïve (CD45RA+) CD31+ T-cells are called recent thymic emigrants (RTE) whereas T-cells that have proliferated in the periphery but without encountering antigen are (CD45RA+) CD31- and called central naïve cells.

7.1.3 Unregulated proliferation

Unregulated T cell proliferation is described in HIV infection due to an imbalance of immune activation and T-cell depletion [459-461]. Whilst ART reduces T-cell

proliferation, abnormally high levels of proliferation can persist despite viral suppression [462], similarly to immune activation. Ki67 is a marker of peripheral T-cell division; with untreated HIV large proportions of cells are actively proliferating which usually falls with effective ART.

To date little work has been published looking at these markers in large populations of HIV-infected children living in Africa. To address this, 2 immunophenotyping panels looking at markers of immune activation (HLA DR and CD38 expression in CD4+ and CD8+ T-cells) and proliferation (Ki67 expression in CD4+ sub populations classified according to CD45RA and CD31) were performed at baseline, week 48 and week 96. Methods of the techniques used are described in section 3. 9.

7.2 Results

7.2.1 Analysis

Markers were truncated at the 1st and 95th centile, to avoid outliers having undue influence on results.

7.2.2 Missing samples

As summarised in Table 7-1, 80-90% of children had available immunophenotying data available at each time point. A few patients did not have data available due either to 1) no sample available or the sample not being processed (difficulties with venesection, no laboratory personnel available or lack of reagents) or 2) due a problem with the processing of the sample such that the results were unanalyzable, specifically:

a) Antibody problem; incorrect antibodies added or one left out (the DR panel was pre-mixed, the Ki67 had to be hand mixed), antibodies damaged by light.

b) Sample processing; left for too long before processing (4 hour window), poor lysis or mixing or ran too fast / slow.

c) Machine problem; incorrect settings used, fluidics flow disturbed by blocked probe, cracked tube, inadequate pressure or loose line.

d) Insufficient events captured; anything with less than 1000 events was not analysed.

All analyses are based on observed data.

Table 7-1. Summary of samples available for immunophenotyping analysis.

			Group 1 HIV infected ART naïve	HIV uninfected matched to group 1	Group 2 HIV infected ART experienced	HIV uninfected matched to group 2
Week 0	DR	**Total**	208	209	74	75
		Samples available	177	162	60	62
		Sample unavailable (%)	31 (15%)	47 (22%)	14 (19%)	13 (17%)
		No sample processed[1]	23	31	11	4
		Sample unanalysable[2]	8	16	3	9
	Ki67	Samples available	187	161	60	63
		Sample unavailable (%)	21 (10%)	48 (23%)	14 (19%)	12 (16%)
		No sample processed[1]	19	32	13	3
		Sample unanalysable[2]	2	16	1	9
Week 48	DR	**Total**	185		74	
		Samples available	161		61	
		Sample unavailable (%)	24 (13%)		13 (18%)	
		No sample processed[1]	20		10	
		Sample unanalysable[2]	4		3	
	Ki67	Samples available	157		63	
		Sample unavailable (%)	28 (15%)		11 (15%)	
		No sample processed[1]	24		10	
		Sample unanalysable[2]	4		1	
Week 96	DR	**Total**	178		74	
		Samples available	141		63	
		Sample unavailable (%)	37 (21%)		11 (15%)	
		No sample processed[1]	35		10	
		Sample unanalysable[2]	2		1	
	Ki67	Samples available	150		62	
		Sample unavailable (%)	28 (16%)		12 (16%)	
		No sample processed[1]	26		11	
		Sample unanalysable[2]	2		1	

(1) No sample processed; insufficient blood taken, no laboratory personnel available to process sample or no reagents available.
(2) Sample unanalysable; failed antibody staining, antibodies damaged by light, delay in processing sample, incorrect settings used or insufficient events (<1000) captured.

7.3 Baseline immunophenotyping results

Markers of activation

Based upon the surface expression of HLA DR and CD38, CD4+ and CD8+ T-cells can be divided into 4 sub-populations, as illustrated in Figure 7-1. Quadrant 2 (Q2) represents the CD38+ HLA DR + or “double positives” that are markers of activated cells; in this example A) shows a HIV uninfected 7 month old infant with 3.35% of his CD4+ T-cells expressing CD38 and HLA DR whilst B) shows a 4 year 10 month old HIV-infected child who is not on ART; he has high levels of activation with 47.8% of his CD4+ cells expressing CD38 and HLA DR.

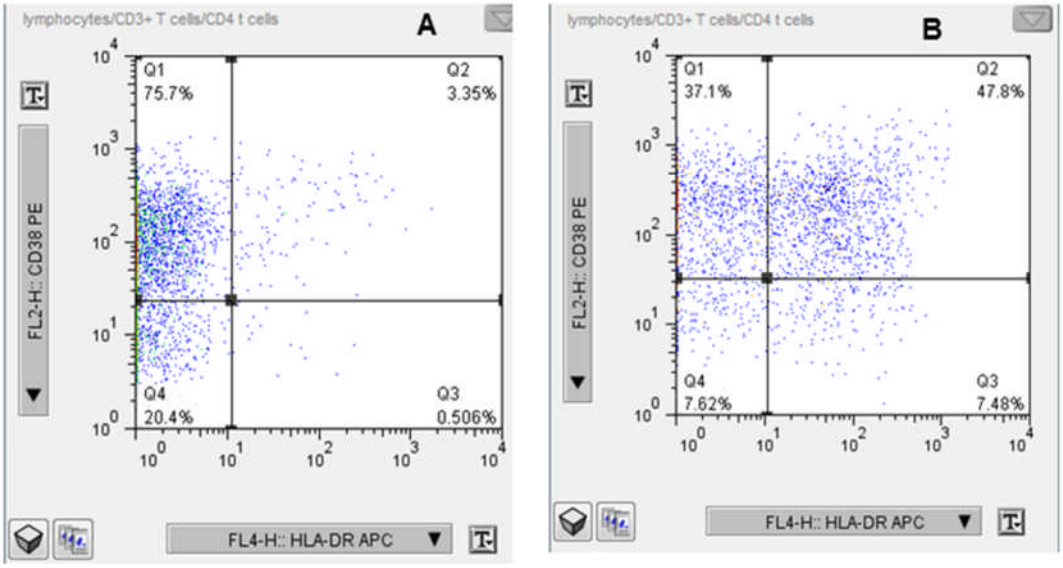


Figure 7-1. CD4+ (and CD8+) T-cells can be divided into 4 groups based upon the surface expression of CD38 and HLA DR.

These examples show the CD4+ population of A) a 7 month old HIV uninfected baby and B) a 4 year 10 month HIV-infected, ART naïve boy. Quadrant 1 (Q1) contains the HLA DR-CD38+ sub population, Q2 contains the HLA DR+CD38+ “double positives”, markers of immune activation. In this example the effects of uncontrolled HIV are clearly demonstrated; in B 47.8% of the child’s CD4+ T-cells are HLA DR+ CD38+ , an highly activated population compared to just 3.35% of the young HIV uninfected infant in A. Q3 contains HLA DR+CD38- and Q4 contains “ double negatives” HLA DR-CD38-.

Results are summarised in Table 7-2 and illustrated in Figure 7-2. The proportion of CD4+ T-cells expressing HLA DR+CD38+ (“double positives”) was significantly higher in HIV-infected, ART naïve children compared to both HIV uninfected controls and HIV-infected, ART experienced children ($p<0.001$). The proportion of CD4 double positives was significantly lower in HIV-infected, ART experienced children compared to controls ($p=0.004$). A similar pattern was seen in the proportion of CD8+ double positives that were significantly higher in HIV-infected ART naïve children compared to both their HIV uninfected controls and HIV-infected ART experienced children ($p<0.001$). There was no evidence of a difference in the proportion of CD8+ double positives in HIV-infected ART experienced children compared to their controls ($p=0.17$).

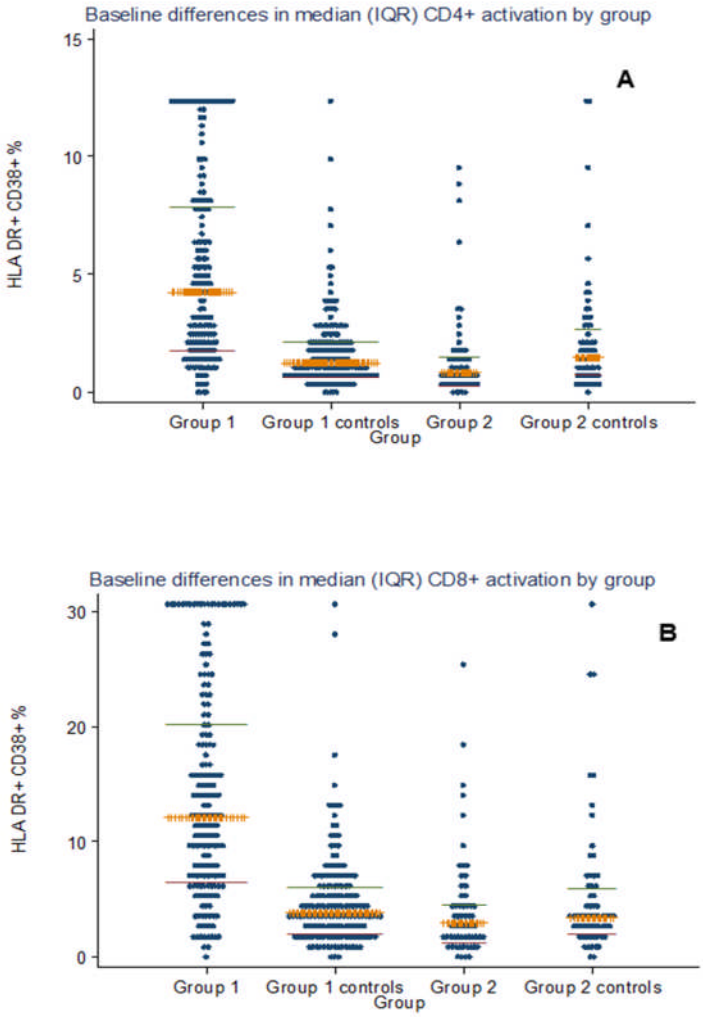


Figure 7-2. Baseline differences in percentage of activated (HLA DR+ CD38+) CD4+ and CD8+ T-cells between groups.

A. *Significant differences are seen in the percentage of activated CD4+ T-cells between HIV-infected, ART naïve (group 1) and controls ($p<0.001$), between HIV-infected ART naïve and HIV-infected ART experienced (group 2) and between group 2 and their HIV uninfected control group ($p<0.001$).*

B. *Significant differences were also seen the percentage of activated CD8+ T-cells between group 1 and controls, between group 1 and group 2 ($p<0.001$) but no significant difference was seen between group 2 and their controls ($p=0.17$).*

Table 7-2. Summary of baseline immunophenotyping results: markers of activation

	HIV infected ART naïve (group 1)	HIV uninfected matched to group 1	difference between group 1 and controls *	HIV infected ART experienced (group 2)	HIV uninfected matched to group 2	difference between group 2 and controls *	difference between group 1 and group 2 *
General							
Number of results	177	162		60	62		
Lymphocytes	27 (18 : 35)	34 (25 : 41)	**p<0.001**	31 (26 : 37)	33 (24 : 38)	p = 0.88	**p = 0.002**
% CD3+	71 (62 : 78)	67 (59 : 72)	**p<0.001**	69 (61 : 73)	67 (61 : 74)	p = 0.40	p = 0.11
% CD4+	16 (11 : 22)	37 (32 : 43)	**p<0.001**	34 (28 : 39)	35 (30 : 44)	p = 0.10	**p < 0.001**
% CD3+ CD8+	61 (53 : 71)	30 (26 : 35)	**p<0.001**	40 (33 : 47)	33 (28 : 39)	**p <0.001**	**p < 0.001**
CD4:CD8	0.45 (0.20 : 0.75)	1.57 (1.03 : 2.19)	**p<0.001**	0.79 (0.56 : 1.35)	1.28 (0.9 : 1.94)	**p <0.001**	**p < 0.001**
Markers of activation							
CD4							
% CD4+ DR- CD38+ (Q1)	83 (68 : 91)	82 (69 : 91)	p=0.95	65 (50 : 80)	66 (56 : 88)	p = 0.34	**p < 0.001**
% CD4+ DR+ CD38+ (Q2)	4.24 (1.9 : 8.0)	1.24 (0.76 : 2.24)	**p<0.001**	0.83 (0.39 : 1.64)	1.51 (0.84 : 2.8)	**p = 0.004**	**p < 0.001**
% CD4+ DR+ CD38- (Q3)	0.25 (0.07 : 0.83)	0.20 (0.08 : 0.51)	p=0.53	0.41 (0.10 : 0.90)	0.34 (0.12 : 1.68)	p = 0.41	p = 0.23
% CD4+ DR- CD38- (Q4)	10 (4 : 22)	17 (7 : 28)	**p<0.001**	32 (17 : 49)	29 (9 : 42)	p = 0.15	**p < 0.001**
CD8							
% CD8+ DR- CD38+ (Q1)	80 (64 : 88)	84 (72 : 91)	**p=0.01**	66 (52 : 82)	72 (55 : 82)	p = 0.59	**p = 0.01**
% CD8+ DR+ CD38+ (Q2)	12.1 (6.7 : 20.4)	3.8 (2.2 : 6.2)	**p<0.001**	2.9 (1.5 : 4.8)	3.4 (2.3 : 6.2)	p = 0.17	**p < 0.001**
% CD8+ DR+ CD38- (Q3)	0.11 (0.02 : 0.56)	0.10 (0.02 : 0.48)	p=0.41	0.20 (0.06 : 0.91)	0.41 (0.13 : 1.28)	p = 0.14	p = 0.09
% CD8+ DR- CD38- (Q4)	4.2 (1.6 : 9.5)	10.5 (4.9 : 21.6)	**p<0.001**	24.7 (12.5 : 43.4)	20.4 (9.9 : 31.9)	p = 0.39	**p < 0.001**

** using Mann Whitney U test. Where a significant difference exists between HIV+ and uninfected controls the higher value is written in red. Where a significant difference exists between ART naïve and ART experienced the higher value is underlined. Q1 - 4 refer to the quadrants illustrated in figure 7.1.*

Cut offs to define low / intermediate / high levels of activation were set arbitrarily at 5% and 10% and the percentage of children in each group, as defined by the proportion of CD4+ and CD8+ T-cells that are expressing both HLA DR+ and CD38+, calculated as summarised in Table 7-3. Of special interest are the small proportion (16%) of HIV-infected, ART naïve children who have low levels of activated CD8+ T-cells and also the 17% / 8% of ART experienced children who remain with medium / high levels of activated CD8+ cells despite a median of 3.7 years of ART and a supressed viral load. However, 24% / 10% of HIV uninfected children had medium / high levels of activated CD8+ cells, suggesting this could be due to non-HIV factors.

Table 7-3. Proportion of children who have low / medium / high levels of activation by group.

	All groups	ART naïve	ART experienced	HIV uninfected
CD4+ HLA DR + CD38 +				
<5 %	371 (80%)	103 (58%)	56 (93%)	212 (95%)
5 - 10%	56 (12%)	44 (25%)	5 (7%)	8 (4%)
≥ 10%	34 (7%)	30 (17%)	0 (0)	4 (2%)
CD8+ HLA DR + CD38 +				
<5 %	223 (48%)	29 (16%)	45 (75%)	149 (67%)
5 - 10%	104 (23%)	41 (23%)	10 (17%)	53 (24%)
≥ 10%	134 (29%)	107 (60%)	5 (8%)	22 (10%)

Markers of proliferation

CD4 T-cells were subdivided into 4 populations based upon the surface expression of CD45RA and CD31 as illustrated in Figure 7-3. The proportions of naïve cells (all CD45RA+) and memory cells (all CD45RA-) were calculated. For each subpopulation the proportion of cells expressing Ki67 was determined; Figure 7-4 illustrates the differences seen in Ki67 expression between HIV-infected children on and off ART. All results are given in Table 7-4.

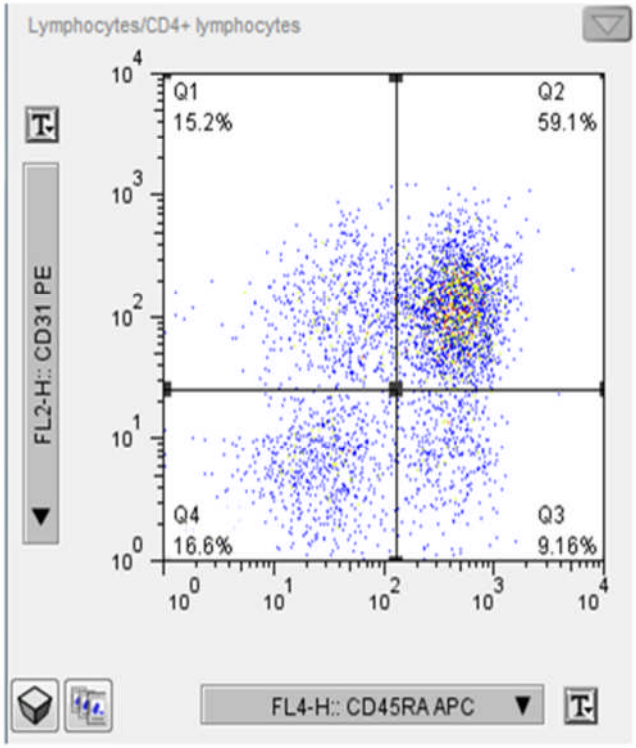


Figure 7-3. CD4+ T-cells can be divided into 4 groups based upon the surface expression of CD45 RA and CD31.

This is an example of a 7 month old HIV-infected baby not on ART. 59.1% of his CD4+ T-cells are recent thymic emigrants expressing CD45 RA+ CD31+ (Quadrant 2)

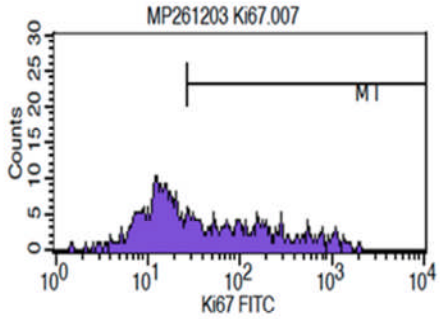
Quadrant 1 (Q1) contains the CD45 RA- CD31+ sub population, these are an abnormal population of memory cells, seen at low frequencies in uninfected children

Q2 contains the CD45 RA+ CD31+ "double positives", recent thymic emigrants (RTE)

Q3 contains CD45 RA+ CD31–; these are central naïve cells

Q4 contains "double negatives" CD45 RA- CD31-; these are true memory cells.

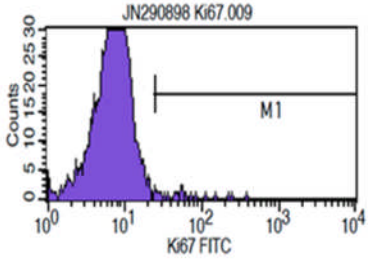
ART naive



File: MP261203 Ki67.007 Acquisition Date: 24-No
Gate: G3 Total Events: 59078

Marker	Events	% Gated	% Total	Median
All	1246	100.00	2.11	22.57
M1	579	46.47	0.98	111.40

ART experienced



File: JN290898 Ki67.009 Acquisition Date: 29-No
Gate: G5 Total Events: 62650

Marker	Events	% Gated	% Total	Mean	Median
All	3700	100.00	5.91	8.02	7.30
M1	25	0.68	0.04	76.97	47.83

Figure 7-4. Examples of Ki67 expression on CD4+ CD45RA- T-cells.

An HIV-infected, ART naïve child who has a 46.5% of his CD4+ CD45RA- T-cells expressing Ki67

An HIV-infected, ART experienced child who has less than 0.7% of CD4+CD45RA- T-cells expressing Ki67

Table 7-4. Summary of baseline immunophenotyping.

Naïve, memory cells and proportion of proliferating by sub-population, median and IQR given.

	HIV infected ART naïve (group 1)	HIV uninfected matched to group 1	difference between group 1 and controls *	HIV infected ART experienced (group 2)	HIV uninfected matched to group 2	difference between group 2 and controls *	difference between group 1 and group 2 *
Markers of proliferation in CD4+ T-cells							
% CD4+ Ki67+	6.4 (3.9 : 9.2)	2.5 (1.6 : 3.7)	**p < 0.001**	3.6 (2.7 : 4.7)	3 (2.2 : 3.9)	**p = 0.02**	**p < 0.001**
Naïve cells							
% CD45 RA+	57.9 (46.6 : 67.8)	69.3 (61.8 : 76.8)	**p < 0.001**	59.0 (51.6 : 67.9)	66.4 (54.4 : 71.7)	**p = 0.01**	p = 0.68
% CD45 RA+ Ki67+	1.9 (1.1 : 4.1)	1.0 (0.6 : 2.3)	**p < 0.001**	1.5 (1.0 : 2.7)	2.4 (0.8 : 3.6)	p = 0.48	p = 0.15
% CD45RA+ CD31+ (Q2)	48 (38 : 60)	60 (52 : 67)	**p < 0.001**	51 (44 : 60)	56 (47 : 62)	p = 0.10	p = 0.21
% CD45RA+ CD31+ Ki67+	1.36 (0.96 : 2.39)	0.94 (0.54 : 1.78)	**p < 0.001**	1.2 (0.75 : 1.54)	1.41 (0.74 : 3.1)	p = 0.35	p = 0.08
% CD45RA+ CD31- (Q3)	8.1 (4.6 : 11.8)	8.6 (6.5 : 11.8)	p = 0.09	5.3 (3.6 : 8.0)	9.6 (5.8 : 12.9)	**p <0.001**	**p = 0.01**
% CD45RA+ CD31- Ki67+	1.55 (0.48 : 4.05)	0.75 (0.35 : 1.92)	**p < 0.001**	1.09 (0.59 : 2.57)	2 (0.54 : 4.0)	p = 0.20	p = 0.22
Memory cells							
% CD45 RA-	41.8 (32.2 : 50.4)	30.6 (23.2 : 38.2)	**p < 0.001**	40.5 (31.6 : 47.4)	33.6 (28.3 : 44.8)	**p = 0.01**	p = 0.62
% CD45 RA- Ki67+	12.2 (8.5 : 16.6)	5.4 (2.8 : 8.0)	**p < 0.001**	6.5 (5.0 : 8.0)	4.9 (3.5 : 7.2)	**p = 0.01**	**p<0.001**
% CD45RA- CD31+ (Q1)	17 (12 : 23)	12 (8 : 17)	**p < 0.001**	17 (10 : 23)	14 (10 : 20)	p = 0.30	p = 0.60
% CD45RA- CD31+ Ki67+	11.9 (7.3 : 16.1)	5 (2.8 : 8.6)	**p < 0.001**	5.6 (4.1 : 7.5)	4.1 (3.3 : 7)	**p = 0.04**	**p < 0.001**
% CD45RA- CD31-(Q4)	22.7 (13.3 : 33.4)	17 (11.9 : 23.3)	**p < 0.001**	23.3 (16.9 : 27.8)	17.9 (11.7 : 23.4)	**p = 0.01**	p = 0.71
% CD45RA- CD31- Ki67+	11.9 (7.2 : 16.6)	4.3 (1.8 : 7)	**p < 0.001**	6.4 (5.0 : 8.2)	4.6 (2.5 : 6.5)	**p <0.001**	**p < 0.001**

*Median and IQR given * using Mann Whitney U test. Where a significant difference exists between HIV+ and uninfected controls the higher value is written in red. Where a significant difference exists between ART naïve and ART experienced the higher value is underlined. Q1 - 4 refer to the quadrants illustrated in figure 7.3.*

Naïve and memory cells

As illustrated in Figure 7-5 and summarised in Table 7-4, HIV-infected ART naïve and experienced children have significantly lower proportions of naïve CD4+ cells compared to their control groups ($p<0.001$). There is no evidence of a difference in the proportion of naïve cells in HIV-infected ART naïve and ART experienced children ($p=0.68$). The HIV-infected ART naïve children have significantly higher percentage of proliferating naïve cells compared to their control group ($p<0.001$). There is no evidence of a difference in proliferating naïve cells between HIV-infected ART experienced and their control group or HIV-infected ART naïve children ($p\geq0.15$).

A similar pattern is seen in the proportions of memory cells; HIV-infected ART naïve and experienced children have significantly higher proportions of memory CD4+ cells compared to their control groups ($p\leq0.01$), whereas there is no evidence of a difference in the proportion of memory cells between HIV-infected ART naïve and ART experienced children ($p=0.62$). The HIV-infected ART naïve and ART experienced children have significantly higher percentage of proliferating memory cells compared to their respective control groups ($p\leq0.01$). HIV-infected ART naïve children have a significantly higher proportion of proliferating memory cells compared to HIV-infected ART experienced children ($p<0.001$).

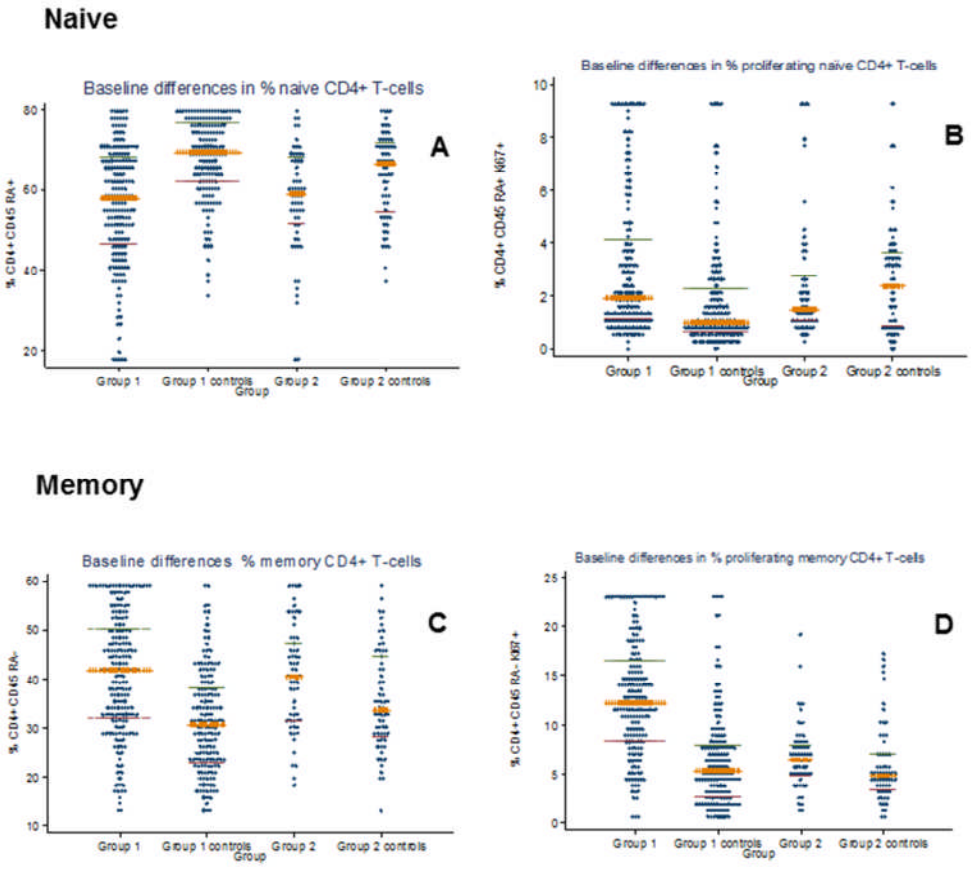


Figure 7-5. Differences in the proportions of subpopulations of naïve and memory cells by group.

A) Naïve CD4+ T-cells

B) Proliferating naïve CD4+ T-cells

C) Memory CD4+ T-cells

D) Proliferating memory CD4+ T-cells

The naïve CD4+ T-cell subpopulations

As illustrated in Figure 7-6, ART naïve children have significantly lower levels of CD31+ naïve (RTEs) cells compared to controls ($p<0.001$), but more similar proportions of central naïve (CD31-) cells to controls ($p=0.09$). However, both the CD31+ and CD31- naive sub populations have significantly higher levels of proliferation compared to controls ($p<0.001$). ART increases the proportion of RTEs to similar levels to controls ($p=0.10$), but in contrast central naïve T-cells are significantly lower than controls ($p<0.001$). In ART experienced children proliferation in the recent thymic emigrants and central naïve cells is similar to uninfected controls ($p\geq0.1$). As overall, there was no evidence of a difference between HIV-infected naïve and ART-experienced children in the proportions of proliferating cells within each naïve subpopulation ($p>0.08$).

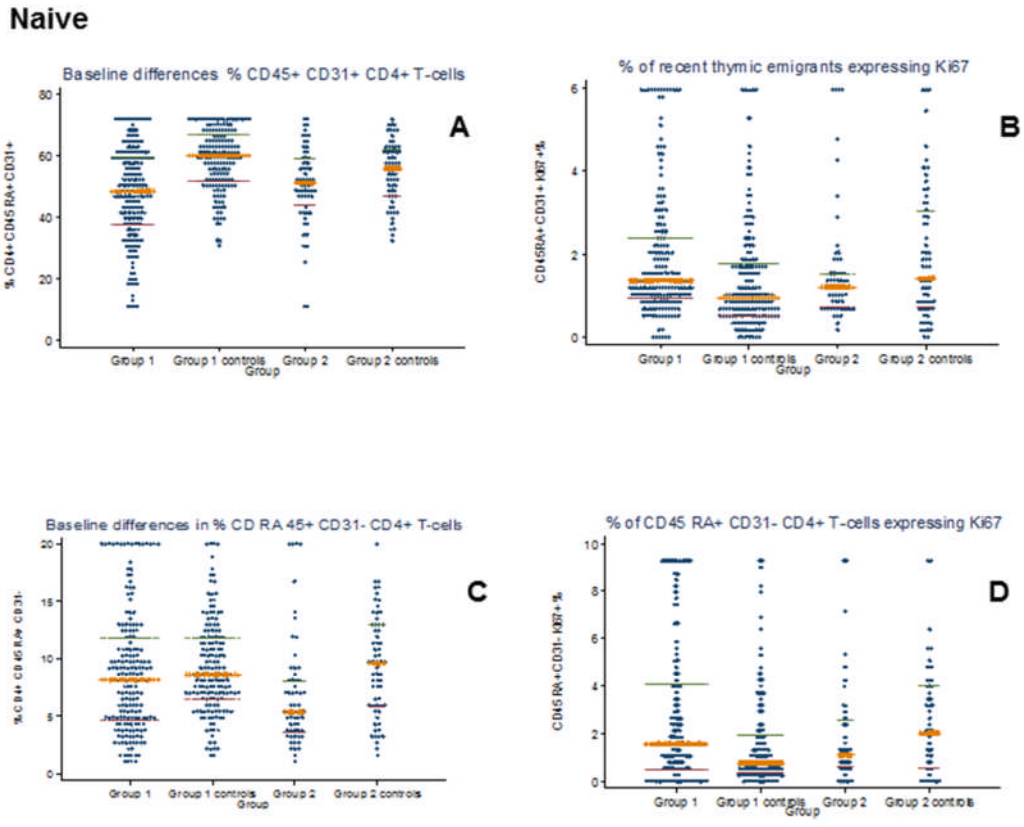


Figure 7-6. Differences by groups of the proportions of sub-populations of the CD4+ CD45RA+ naïve T-cells and proliferating cells.

A) Total recent thymic emigrants

B) Proliferating recent thymic emigrants

C) Total central naïve cells

D) Proliferating central naïve cells.

The memory CD4+ T-cell subpopulations

As illustrated in Figure 7-7, the CD45RA- CD31+ subpopulation is significantly larger in HIV-infected, ART naïve children compared to their control group ($p<0.001$). No significant differences are seen between HIV-infected ART experienced and their control group nor between HIV-infected children on and off ART ($p\geq0.30$). The proportions of CD45-CD31+ expressing Ki67 were significantly higher in HIV-infected, ART naïve children compared to their control group, HIV-infected ART experienced compared to their control group and in HIV-infected ART naïve compared to ART experienced ($p\leq0.04$).

Proportions of the true memory population (CD45RA- CD31-) were significantly higher in HIV-infected ART naïve and ART experienced children compared to their respective control groups ($p<0.001$). No significant differences were seen between HIV-infected, ART naïve and ART experienced children ($p=0.71$). The proportion of true memory cells expressing Ki67 was significantly higher in HIV-infected, ART naïve compared to controls and to HIV-infected, ART experienced; additionally the proportion expressing Ki67 was also higher in HIV-infected, ART experienced compared to their control group (all $p<0.001$).

Memory

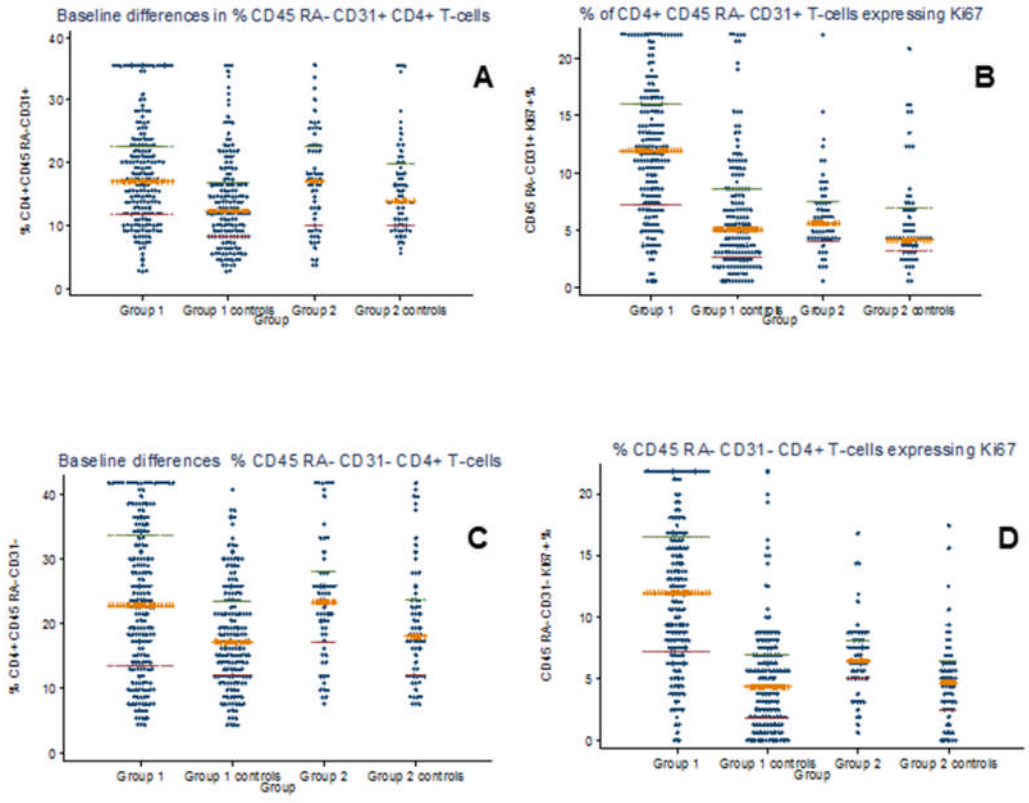


Figure 7-7. Differences between groups of the proportions of different sub-populations of the CD4+ CD45RA- memory T-cells and the proportions of proliferating cells.

A) CD45 RA- CD31+ T-cells

B) Proliferating CD45 RA- CD31+ T-cells

C) CD45 RA-CD31- T-cells

D) Proliferating CD45 RA-CD31- T-cells

Table 7-5 summarises overall and by group the proportion of children with low / medium / high levels of proliferating CD4+ subpopulations, categorised by the CD45 RA, CD31 and Ki67 expression using arbitrary thresholds.

Table 7-5. Proportions of children with low / medium / high levels of proliferating CD4+ naïve and memory sub populations.

	All groups	ART naïve	ART experienced	HIV uninfected
NAÏVE T-CELLS				
CD45RA+ CD31+				
<25 %	20 (4%)	17 (9%)	3 (5%)	
25 - 50%	158 (34%)	85 (45%)	25 (42%)	48 (21%)
≥ 50%	293 (62%)	85 (45%)	32 (53%)	176 (79%)
CD45RA+ CD31+ Ki67+				
<2.5 %	372 (79%)	141 (75%)	52 (87%)	179 (80%)
2.5 - 5%	68 (14%)	29 (16%)	4 (7%)	35 (16%)
≥ 5%	31 (7%)	17 (9%)	4 (7%)	10 (5%)
CD45RA+ CD31-				
<5 %	112 (24%)	55 (29%)	24 (40%)	33 (15%)
5 - 10%	200 (42%)	72 (39%)	26 (43%)	102 (46%)
≥ 10%	159 (34%)	60 (32%)	10 (17%)	89 (40%)
CD45RA+ CD31- Ki67+				
<2.5 %	327 (69%)	117 (63%)	44 (73%)	166 (74%)
2.5 - 5%	82 (17%)	31 (17%)	11 (18%)	40 (18%)
≥ 5%	62 (13%)	39 (21%)	5 (8)	18 (8%)
MEMORY T-CELLS				
CD45RA- CD31+				
<10 %	127 (27%)	34 (18%)	15 (25%)	78 (35%)
10 - 20%	213 (45%)	86 (46%)	24 (40%)	103 (46%)
≥ 20%	131 (28%)	67 (36%)	21 (35%)	43 (19%)
CD45RA- CD31+ Ki67+				
<5 %	162 (34%)	21 (11%)	24 (40%)	117 (52%)
5 - 10%	142 (30%)	47 (25%)	30 (50%)	65 (29%)
≥ 10%	167 (35%)	119 (64%)	6 (10%)	42 (19%)
CD45RA- CD31-				
<10 %	71 (15%)	31 (17%)	5 (8%)	35 (16%)
10 - 20%	173 (37%)	51 (28%)	15 (25%)	107 (48%)
≥ 20%	227 (48%)	105 (56%)	40 (67%)	82 (37%)
CD45RA- CD31- Ki67+				
<5 %	163 (35%)	26 (14%)	13 (22%)	124 (55%)
5 - 10%	179 (38%)	52 (28%)	42 (70%)	85 (38%)
≥ 10%	129 (27%)	109 (58%)	5 (8%)	15 (7%)

7.4 The ratio of CD4 to CD8 cells.

A healthy CD4:CD8 ratio in children is generally considered to be greater than 1. A reversal of the ratio is seen with CD4+ T-cell depletion or CD8 expansion, causes of which include infection with HIV, EBV or CMV and other conditions including malignancies. As summarised in Table 7-6, the majority (77%) of ART naïve children had a CD4:CD8 ratio of less than one reflecting their low CD4 count. Despite a median of 3.7 years on ART 49% of ART experienced children still had a ratio under one, predominantly reflecting high CD8 counts as their CD4s were good, and surprisingly 21% of "normal" healthy children had a low ratio; 25/60 of these children had a low CD4 count as described in section 5. 2 whilst in the remaining 35/60 this was due to CD8 expansion possibly reflecting current EBV or CMV infections, both extremely common in young African children. Within the population of ART experienced children, there was no evidence that either prior duration of ART or age at starting ART were associated with a CD4:CD8 ratio of under one ($p \geq 0.29$).

Table 7-6. Summary of the ratio of CD4:CD8 T-cells at baseline overall and by group.

	All groups	ART naïve	ART experienced	HIV uninfected
CD4 : CD8				
< 1	256 (45%)	160 (77%)	36 (49%)	60 (21%)
>1	310 (55%)	48 (23%)	38 (51%)	224 (79%)

7.5 Effect of age, CD4 and viral load on baseline immunophenotyping results

7.5.1 Activation

There was no convincing evidence of a relationship between viral load, age, CD4 count, CD4 percentage or CD4 z score and activated (HLA DR+ CD38+) CD4+ and CD8+ T-cells in the separate groups of HIV-infected, ART experienced or HIV uninfected children (p≥0.05,Table 7-7).

Table 7-7. Relationship of baseline viral load, age and CD4 count / CD4 percentage and CD4 z score with percentage of activated CD4+ and CD8+ T-cells.

		Viral load		**Age**		**CD4**		**CD4 percentage**		**CD4 z score**	
		r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
CD4+ HLA DR+ CD38+	ART naïve	0.14	0.07	0.08	0.29	-0.14	0.07	-0.13	0.09	-0.13	**0.05**
	ART experienced	NA	NA	-0.09	0.48	0.08	0.55	-0.07	0.57	0.06	0.63
	HIV uninfected	NA	NA	0.12	0.07	-0.10	0.14	-0.08	0.24	-0.01	0.90
CD8+ HLA DR+ CD38+	ART naïve	0.01	0.88	0.01	0.88	0.01	0.92	0.10	0.19	0.01	0.87
	ART experienced	NA	NA	-0.08	0.53	0.12	0.38	-0.06	0.65	0.11	0.40
	HIV uninfected	NA	NA	0.01	0.83	-0.01	0.87	-0.03	0.66	0.01	0.84

In the HIV-infected, ART naïve children, there was a trend towards a lower percentage of CD4+ HLA DR+ CD38+ expressing CD4+ T-cells with increasing CD4 / CD4 percentage / CD4 z score but this was weak in magnitude and did not reach significance (r -0.14 / -0.13 / -0.13), (p=0.07 / 0.09 / 0.05) (Figure 7-8).

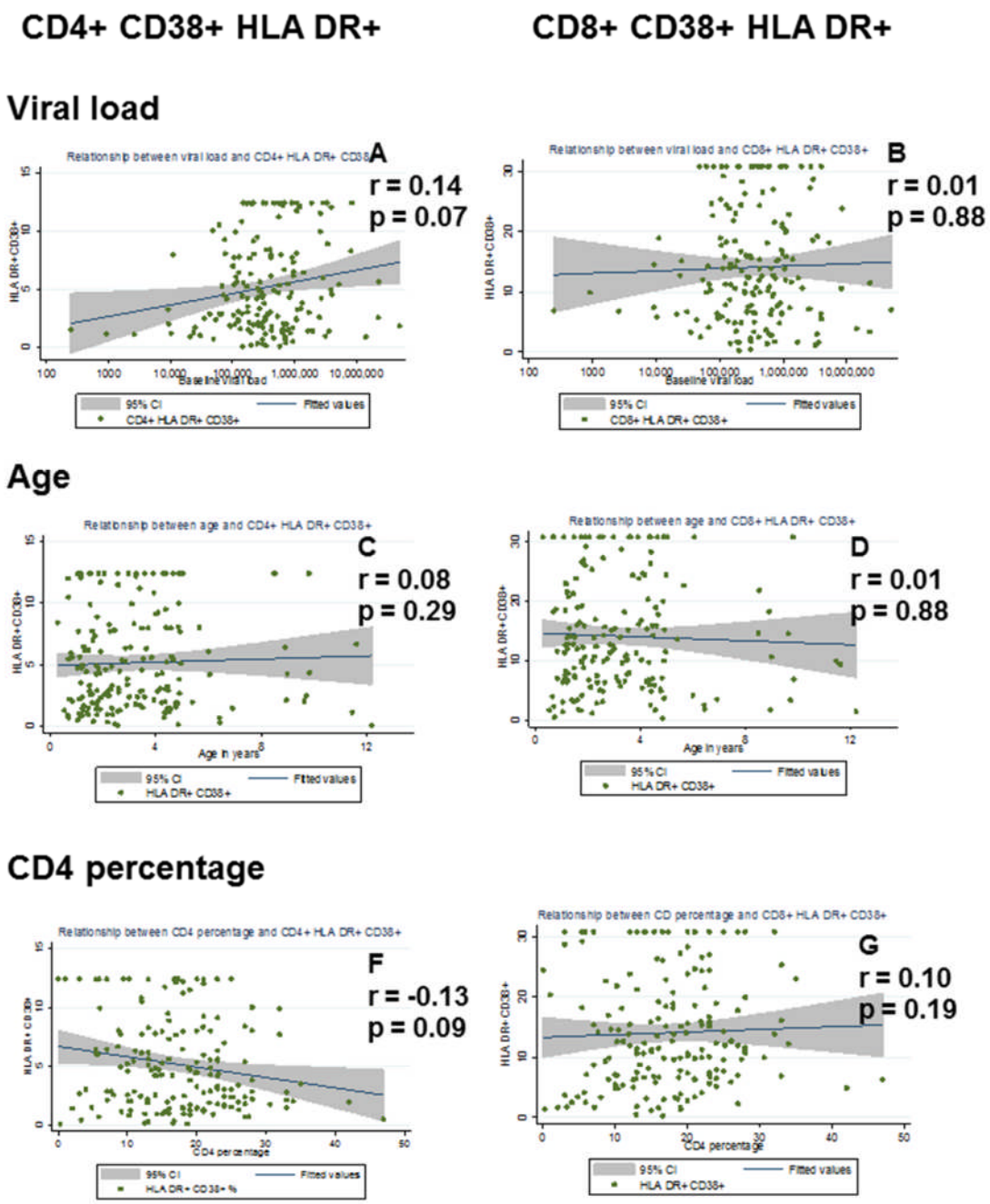


Figure 7-8. Influence of baseline viral load, age and CD4 percentage on markers of activation in HIV-infected, ART naïve children.

The strength of the correlations was determined by Spearman's rank coefficient (r) and the level of statistical significance given (p).

7.5.2 Proliferation

The relationship between the proportions of total naïve and memory CD4+ T-cells (based upon the surface expression of CD45RA and CD31) and the proportion that was proliferating (expressing Ki67) and baseline viral load, age and CD4 count are presented in Table 7-8.

Naïve T-cells

No significant relationship between baseline viral load and proportions of naïve T-cells subpopulations or level of proliferation was demonstrated in HIV-infected naïve children.

Age was significantly inversely related to the proportion of recent thymic emigrants (CD45RA+ CD31+) in ART naive and HIV uninfected children ($p<0.001$). An inverse relationship was also seen in HIV-infected naïve children between age and the proportion of central naïve cells (CD45RA+ CD31-) ($p=0.03$), with a similar magnitude of negative association in the smaller number of HIV-infected ART-experienced children ($p=0.20$). Proliferating RTEs and proliferating central naïve cells were higher with increasing age in HIV uninfected controls ($p=0.001$), but were not associated with age in either HIV-infected group ($p>0.1$).

Proportions of RTEs increased with increasing CD4 percentage in all groups ($p\leq 0.03$), but there was no association between central naïve proportions and CD4 percentage in any group ($p>0.1$). CD4 percentage and the proportion of proliferating RTEs and central naïve cells were inversely associated in ART naïve and HIV uninfected children ($p<0.001$), but there was no evidence of any relationship in HIV-infected children on ART ($p>0.7$).

Memory T-cells

No significant relationship between baseline viral load and proportions of memory T-cells subpopulations or level of proliferation was demonstrated ($p>0.06$).

Age was significantly associated with proportions of true memory cells (CD45RA-CD31-) in HIV naïve and HIV uninfected children ($p\leq 0.01$) with a similar magnitude of negative association in the smaller number of HIV-infected ART-experienced

children (p=0.21). In HIV uninfected children age was also significantly associated with the proportion of CD31+ memory cells (p=0.02) with a similar trend in HIV-infected naïve children (p=0.05). Lower levels of proliferation in CD31+ memory cells were associated with increasing age in HIV uninfected children only.

CD4 percentages were significantly inversely related to the proportion of CD31+ and CD31- memory cells in HIV uninfected and HIV-infected ART-naive children ($p \leq 0.04$), with a similar trend in ART experienced (p=0.07, 0.10). Higher CD4 percentages were also associated with decreased proliferation in these subpopulations in ART naïve children and in HIV uninfected children ($p \leq 0.05$).

Table 7-8. Relationship between the proportion of total and proliferating CD4+ sub-populations (based upon expression of CD45 RA and CD31) and viral load, age and CD4 percentage.

		Viral load		Age		CD4 percentage	
		r	p	r	p	r	p
NAÏVE T-CELLS							
CD45RA+ CD31+	ART naïve	0.05	0.55	**-0.30**	**<0.001**	**0.48**	**<0.001**
	ART experienced	NA	NA	-0.11	0.42	**0.28**	**0.03**
	HIV uninfected	NA	NA	**-0.24**	**<0.001**	**0.32**	**<0.001**
CD45RA+ CD31+ Ki67+	ART naïve	-0.04	0.58	0.11	0.12	**-0.28**	**<0.001**
	ART experienced	NA	NA	-0.03	0.79	-0.04	0.75
	HIV uninfected	NA	NA	**0.18**	**0.001**	**-0.27**	**<0.001**
CD45RA+ CD31-	ART naïve	0.12	0.10	**-0.16**	**0.03**	-0.11	0.13
	ART experienced	NA	NA	-0.17	0.20	0.06	0.64
	HIV uninfected	NA	NA	0.03	0.74	-0.06	0.38
CD45RA+ CD31- Ki67+	ART naïve	-0.04	0.55	0.02	0.76	**-0.17**	**0.02**
	ART experienced	NA	NA	-0.09	0.49	0.05	0.70
	HIV uninfected	NA	NA	**0.21**	**0.001**	**-0.23**	**0.001**
MEMORY T-CELLS							
CD45RA- CD31+	ART naïve	0.14	0.06	0.14	0.06	**-0.26**	**<0.001**
	ART experienced	NA	NA	0.09	0.48	-0.24	0.07
	HIV uninfected	NA	NA	**0.16**	**0.02**	**-0.14**	**0.04**
CD45RA- CD31+ Ki67+	ART naïve	0.09	0.25	0.01	0.90	**-0.15**	**0.04**
	ART experienced	NA	NA	0.03	0.80	0.06	0.66
	HIV uninfected	NA	NA	**-0.14**	**0.03**	-0.13	0.05
CD45RA- CD31-	ART naïve	-0.08	0.31	**0.30**	**<0.001**	**-0.29**	**<0.001**
	ART experienced	NA	NA	0.17	0.21	-0.22	0.10
	HIV uninfected	NA	NA	**0.17**	**0.01**	**-0.22**	**0.001**
CD45RA- CD31- Ki67+	ART naïve	0.04	0.57	-0.01	0.87	**-0.17**	**0.02**
	ART experienced	NA	NA	-0.07	0.59	0.05	0.72
	HIV uninfected	NA	NA	0.00	0.93	**-0.20**	**0.003**

As discussed above both age and CD4 percentage were significantly related, on univariate analysis, to many of the proliferating CD4+ subpopulations. To determine whether either one was a more important predictor a multivariate analysis was performed - Table 7-9 . Of interest for CD45RA+CD31+ / CD45RA+CD31+ Ki67+ / CD45RA+CD31- Ki67+ / CD45RA-CD31- subpopulations both age and CD4 percentage were independently significant predictors in both HIV infected, ART naïve and HIV uninfected children ($p \leq 0.05$). Within the CD45RA- CD31+ subpopulation age and CD4 percentage were both significant predictors in HIV uninfected children ($p=0.01$), whilst in the ART naïve children only CD4 percentage was significant ($p=0.001$). Age was significantly related to the proportion of CD45RA+CD31- cells in HIV infected ART naïve and ART experienced children ($p \leq 0.05$). CD4 percentage was a significant predictor of CD45RA-CD31+Ki67+ ad CD45RA-CD31-Ki67+ subpopulations in the HIV uninfected children ($p \leq 0.04$).

Table 7-9. Multi-variable analysis exploring the relationship between the proportion of total and proliferating CD4+ sub-populations (based upon expression of CD45 RA and CD31) age and CD4 percentage.

		Age				CD4 percentage			
		co-efficient	95% CI		p value	co-efficient	95% CI		p value
NAÏVE T-CELLS									
CD45RA+ CD31+	ART naïve	-0.02	-0.03	-0.01	**0.001**	0.01	0.01	0.01	**<0.001**
	ART experienced	0.00	-0.03	0.02	0.71	0.00	0.00	0.01	0.23
	HIV uninfected	-0.01	-0.01	0.00	**<0.001**	0.00	0.00	0.01	**<0.001**
CD45RA+ CD31+ Ki67+	ART naïve	0.02	0.00	0.04	**0.03**	-0.01	-0.02	-0.01	**<0.001**
	ART experienced	-0.01	-0.05	0.04	0.74	0.00	-0.01	0.01	1.00
	HIV uninfected	0.03	0.01	0.05	**0.002**	-0.02	-0.03	-0.01	**<0.001**
CD45RA+ CD31-	ART naïve	-0.02	-0.04	0.00	**0.03**	0.00	-0.01	0.00	0.14
	ART experienced	-0.04	-0.08	0.00	**0.05**	0.00	0.00	0.01	0.38
	HIV uninfected	0.00	-0.01	0.01	1.00	0.00	-0.01	0.00	0.27
CD45RA+ CD31- Ki67+	ART naïve	0.04	0.01	0.06	**0.003**	-0.02	-0.03	-0.01	**<0.001**
	ART experienced	-0.02	-0.08	0.05	0.63	0.01	-0.01	0.02	0.31
	HIV uninfected	0.05	0.02	0.07	**<0.001**	-0.02	-0.03	-0.01	**<0.001**
MEMORY T-CELLS									
CD45RA- CD31+	ART naïve	0.01	0.00	0.02	0.15	-0.01	-0.01	0.00	**0.001**
	ART experienced	0.02	-0.02	0.05	0.30	-0.01	-0.01	0.00	0.08
	HIV uninfected	0.02	0.00	0.03	**0.01**	-0.01	-0.01	0.00	**0.01**
CD45RA- CD31+ Ki67+	ART naïve	0.00	-0.02	0.02	0.92	0.00	-0.01	0.00	0.34
	ART experienced	0.00	-0.03	0.04	0.84	0.00	-0.01	0.01	0.92
	HIV uninfected	-0.02	-0.03	0.00	0.08	-0.01	-0.01	0.00	**0.04**
CD45RA- CD31-	ART naïve	0.02	0.01	0.04	**0.002**	-0.01	-0.01	0.00	**0.004**
	ART experienced	0.00	-0.02	0.03	0.76	-0.01	-0.01	0.00	0.07
	HIV uninfected	0.02	0.01	0.03	**0.001**	-0.01	-0.01	0.00	**<0.001**
CD45RA- CD31- Ki67+	ART naïve	0.01	-0.01	0.03	0.20	0.00	-0.01	0.00	0.19
	ART experienced	-0.01	-0.05	0.02	0.39	0.00	-0.01	0.01	0.98
	HIV uninfected	0.01	-0.02	0.03	0.60	-0.01	-0.02	0.00	**0.01**

7.5.3 Effects of age on percentage of recent thymic emigrants

In HIV-infected, ART naïve and HIV uninfected children a significant negative correlation between age and proportion of CD4+ cells that were recent thymic emigrants was demonstrated ($p<0.001$). In the smaller number of ART experienced children there was no evidence of an association ($p=0.42$) - Figure 7-9.

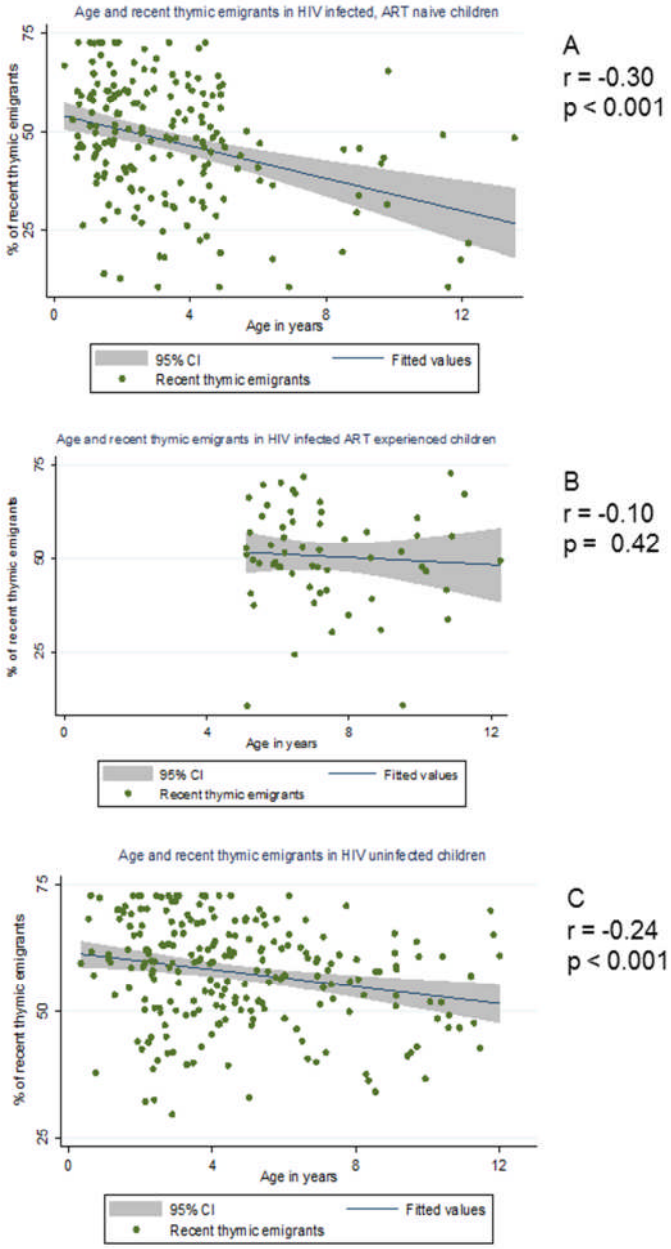


Figure 7-9. Effect of age on percentage of recent thymic emigrants

A) HIV-infected, ART naïve

B) HIV-infected ART experienced

C) HIV uninfected control children.

A significant inverse relationship between age and proportion of recent thymic emigrants was demonstrated in the HIV-infected ART naïve children and the HIV uninfected control group. No significant effect was seen in the (older) HIV-infected, ART experienced children.

7.5.4 The relationship between markers of activation and proliferation

The degree of association between markers of activation (HLA DR and CD38) in CD4+ and CD8+ T-cells with the total and proliferating proportions of naïve and memory CD31+ and CD31- subpopulations was calculated, Table 7-10.

In the HIV-infected naïve and uninfected groups there were no associations between any naïve subpopulation, or proliferation in any naïve sub-population, and levels of activation in CD4+ and CD8+ T-cells ($p>0.05$). The only significant association in the HIV-infected ART-experienced was an inverse relationship between CD31- naive (central naïve) T-cells and activation in CD4+ T-cells ($p=0.003$).

In the memory population, interestingly the most consistent significant relationship were between CD31+ memory T-cells and activation in both CD4+ and CD8+ T-cells in the HIV-infected naïve group ($p\leq0.03$). ART-experienced children with higher proportions of CD31+ memory T-cells also had higher CD4+ activation ($p=0.02$).

Table 7-10. Correlation between markers of activation (HLA DR and CD38) in CD4+ and CD8+ T-cells with expression of Ki67 as a marker of proliferation in 4 sub-populations of CD4+ T-cells based upon their expression of CD45RA and CD31.

		CD4+ HLA DR+ CD38+		CD8+ HLA DR+ CD38+	
		r	**p**	**r**	**p**
NAÏVE					
CD45RA+ CD31+	ART naïve	-0.14	0.06	0.00	0.97
	ART experienced	-0.11	0.42	-0.10	0.43
	HIV uninfected	-0.09	0.20	-0.09	0.20
CD45RA+ CD31+ Ki67+	ART naïve	-0.07	0.34	-0.08	0.27
	ART experienced	0.14	0.27	-0.01	0.93
	HIV uninfected	-0.04	0.56	0.03	0.69
CD45RA+ CD31-	ART naïve	0.05	0.53	0.04	0.63
	ART experienced	**-0.37**	**0.003**	0.01	0.97
	HIV uninfected	-0.02	0.81	0.00	1.00
CD45RA+ CD31- Ki67+	ART naïve	-0.09	0.24	-0.02	0.76
	ART experienced	-0.19	0.14	0.10	0.43
	HIV uninfected	-0.05	0.42	0.07	0.33
MEMORY					
CD45RA- CD31+	ART naïve	0.05	0.51	-0.10	0.20
	ART experienced	**0.31**	**0.02**	0.06	0.67
	HIV uninfected	0.05	0.45	0.00	0.97
CD45RA- CD31+ Ki67+	ART naïve	**0.23**	**0.002**	**0.16**	**0.03**
	ART experienced	0.01	0.96	0.09	0.49
	HIV uninfected	0.00	0.97	0.01	0.87
CD45RA- CD31-	ART naïve	0.12	0.11	0.07	0.38
	ART experienced	-0.04	0.74	0.05	0.68
	HIV uninfected	0.08	0.25	**0.14**	**0.04**
CD45RA- CD31- Ki67+	ART naïve	0.11	0.16	0.15	0.05
	ART experienced	-0.17	0.18	0.20	0.13
	HIV uninfected	-0.04	0.55	0.08	0.24

7.5.5 Relationship between immunophenotyping data, vascular function and biomarkers.

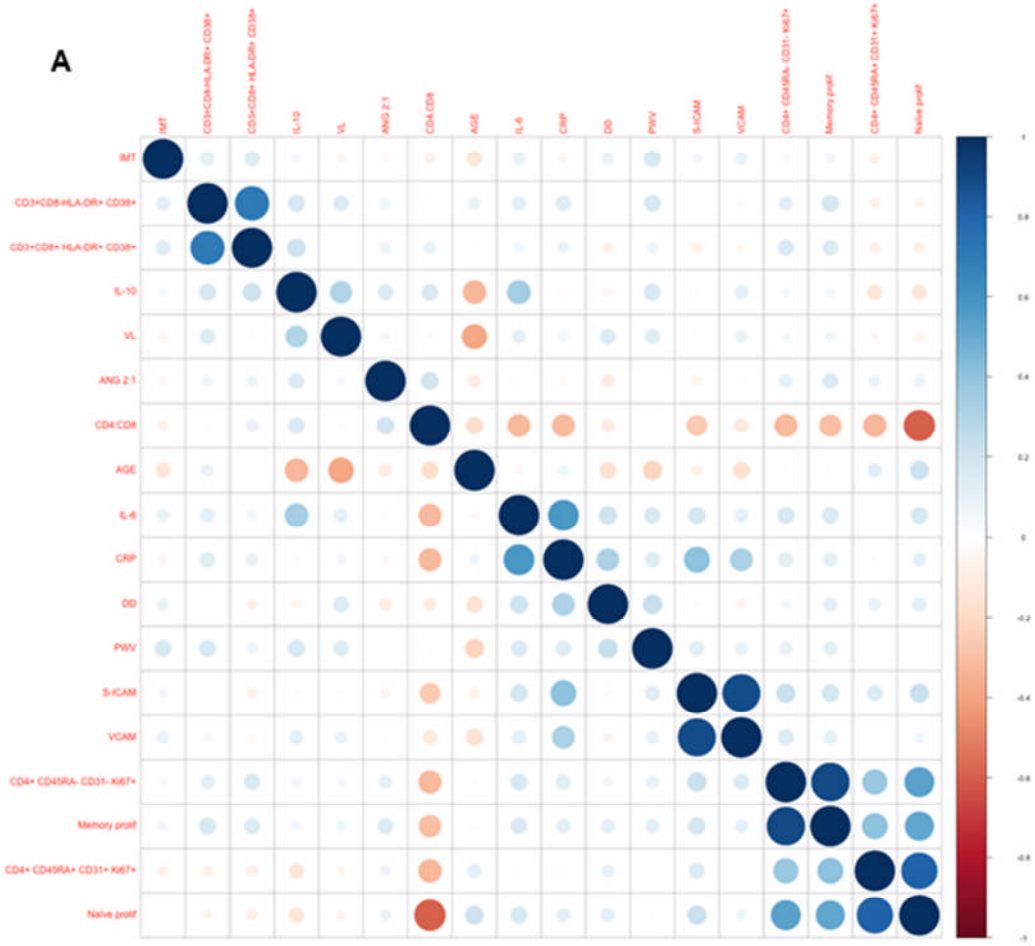
Associations between immunophenotyping data, biomarkers, viral load, IMT and PWV were investigated in each group (ART naïve, ART experienced and HIV uninfected) at baseline using univariable Spearman correlation coefficients.

ART naive

All correlations between the immunophenotyping data and biomarkers, viral load, age, IMT or PWV were <0.35 in magnitude. As illustrated in Figure 7-10, significant correlations exist at baseline between:

- The 2 markers of activation CD4+ HLADR+ CD38+ and CD8+ HLADR+ CD38+ (correlation coefficient r=0.71).
- Within the population of proliferating CD4+ cells, the proportion of CD45RA-CD31-Ki67+ cells positively correlated with the total proportion of proliferating memory cells (r=0.9), the total proportion of proliferating naïve cells (r=0.54) and to a lesser extent the proportion of CD45RA+CD31+Ki67+ cells (r=0.38).
- The proportion of total CD4+ memory cells that were proliferating was significantly correlated with the proportion of naïve cells that were proliferating (r=0.51) and the percentage of CD4+CD45RA+CD31+Ki67+ cells (r=0.4).
- The proportion of CD4+ naïve proliferating cells was significantly correlated with the CD4+CD45RA+CD31+Ki67+ population (r=0.8).

The ratio of CD4:CD8 had a significant negative correlation with the total proportion of proliferating naïve cells (r=-0.59), all other correlations were <0.35. Relationships between age, viral load and biomarkers were as described in chapter 6.


A
IMT
CD3+CD8-HLA-DR+ CD38+
CD3+CD8+ HLA-DR+ CD38+
IL-10
VL
ANG 2:1
CD4:CD8
AGE
IL-6
CRP
DD
PWV
S-ICAM
VCAM
CD4+ CD45RA- CD31- Ki67+
Memory prolif
CD4+ CD45RA+ CD31+ Ki67+
Naive prolif

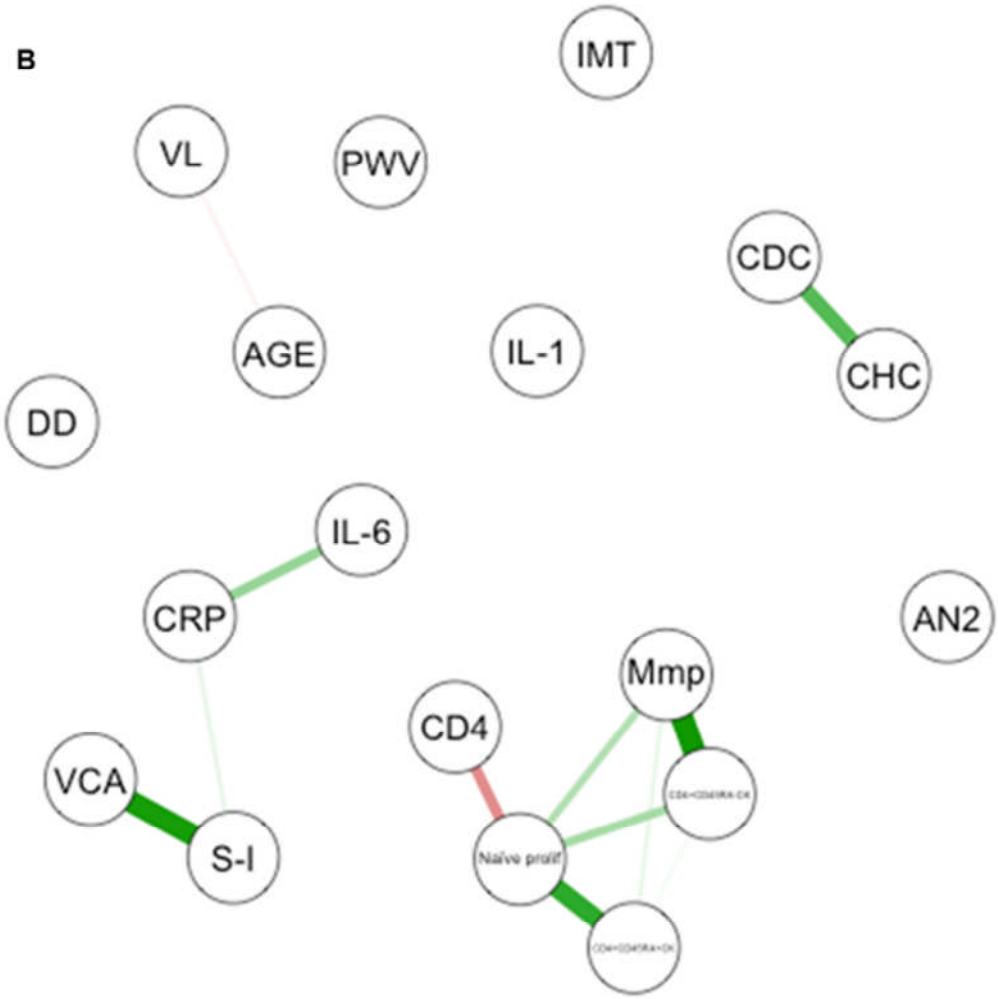


Figure 7-10. Relationship between immunophenotyping, biomarkers, viral load, age, IMT and PWV at baseline in ART naïve HIV-infected children.

In the correlation matrix (A) the strength of positive relationships are demonstrated using blue scale and negative correlations on the red scale. In the nodal diagram (B) relationships with a spearman correlation of >0.35 are demonstrated, positive correlations shown in green and negative correlations in red.

AN2, ANG2:1, ratio of angiopoietin-2:1 CD4, CD4:CD8, CDC, CD3+CD8-HLA DR+ CD38+, CHC, CD3+CD8+HLA DR+ CD38+, CRP, C reactive protein; DD, d-dimer; IL-10, interleukin-10; IL-6, interleukin-6; SI, IMT, intimal medial thickness; Mmp, proportion of memory cells that are proliferating; naïve prolif, proportion of naïve cells that are proliferating PWV, pulse wave velocity; sICAM, soluble intracellular adhesion molecule; VCA, soluble vascular cell adhesion molecule-1; VL, viral load;

ART experienced

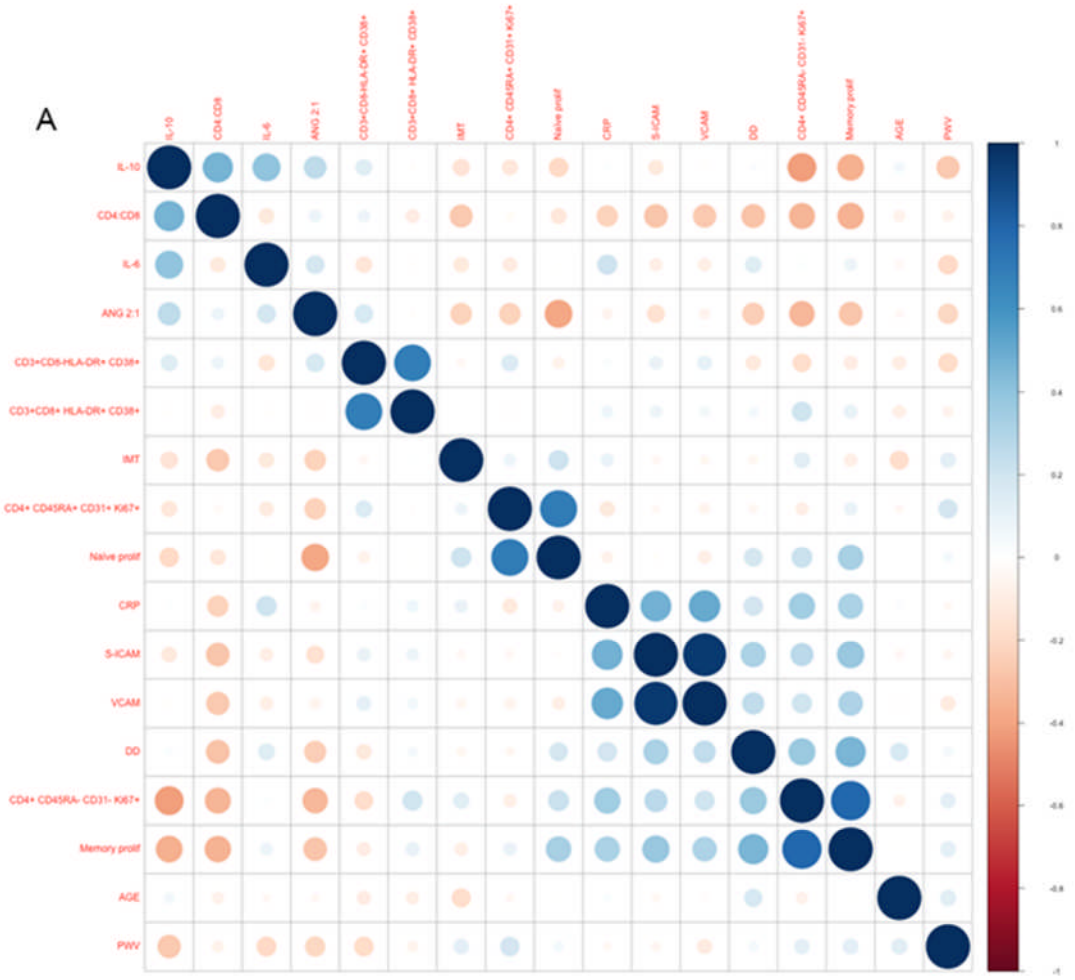
As illustrated in Figure 7-11, similar to the patterns seen in ART naïve children significant correlations exist between:

- the 2 markers of activation CD4+ HLADR+ CD38+ and CD8+ HLADR+ CD38+ (r=0.68),
- CD4+CD45RA-CD31-Ki67+ cells and the total proportion of proliferating CD4+ memory cells (r=0.78)
- CD4+CD45RA+CD31+Ki67+ and the total number of naïve CD4+ cells that were proliferating (r=0.7)

Interestingly there were no significant correlation between immunophenotyping data, IMT, PWV and age (r all≤0.35).

Within this group stronger correlations were seen between some immunophenotyping data and the biomarkers

- The proportion of proliferating CD4+ naïve cells had an inverse relationship with the Angiopoietin 2:1 ratio (r=-0.38)
- The proportion of CD4+CD45RA-CD31-Ki67+ was inversely correlated with IL-10 (r=-0.42) and positively correlated with d-dimer (r=0.37)
- The proportion of proliferating CD4+ memory cells was negatively correlated with IL-10 (r=-0.36) and positively correlated with sICAM (r=0.37) and d-dimer (r=0.46)
- The CD4:8 ratio was positively correlated with IL-10 (r=0.46)


A
IL-10
CD4:CD8
IL-6
ANG 2:1
CD3+CD8-HLA-DR+ CD38+
CD3+CD8+ HLA-DR+ CD38+
IMT
CD4+ CD45RA+ CD31+ Ki67+
Naïve prolif
CRP
S-ICAM
VCAM
DD
CD4+ CD45RA- CD31- Ki67+
Memory prolif
AGE
PWV

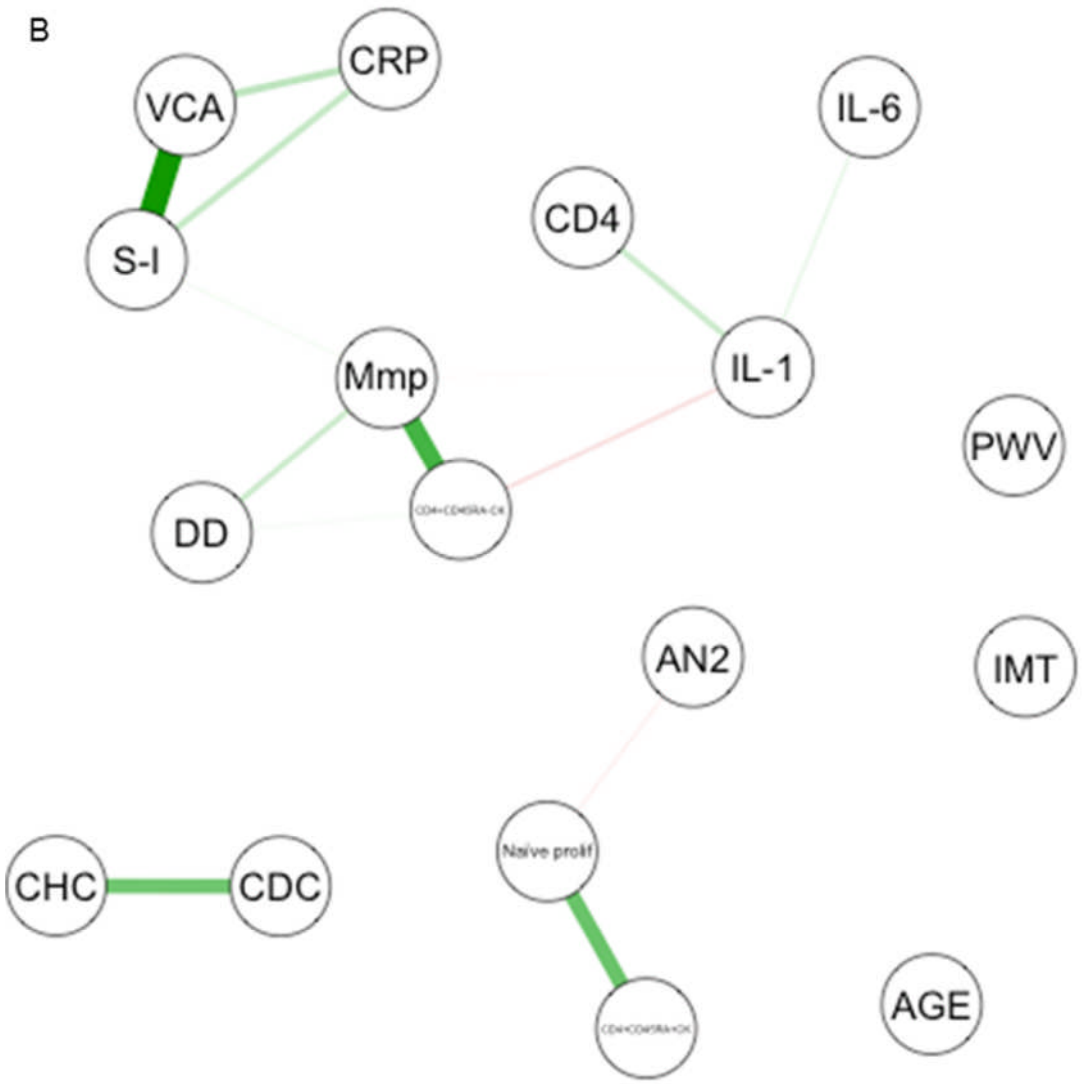


Figure 7-11. Relationship between immunophenotyping, biomarkers, viral load, age, IMT and PWV at baseline in ART experienced HIV-infected children.

In the correlation matrix (A) the strength of positive relationships are demonstrated using blue scale and negative correlations on the red scale. In the nodal diagram (B) relationships with a spearman correlation of >0.35 are demonstrated, positive correlations shown in green and negative correlations in red.

AN2, = ANG2:1, ratio of angiopoietin-2:1 CD4, CD4:CD8, CDC, CD3+CD8-HLA DR+ CD38+, CHC, CD3+CD8+HLA DR+ CD38+, CRP, C reactive protein; DD, d-dimer; IL-10, interleukin-10; IL-6, interleukin-6; SI, IMT, intimal medial thickness; Mmp, proportion of memory cells that are proliferating; naïve prolif, proportion of naïve cells that are proliferating PWV, pulse wave velocity; sICAM, soluble intracellular adhesion molecule; VCA, soluble vascular cell adhesion molecule-1; VL, viral load;

HIV uninfected controls

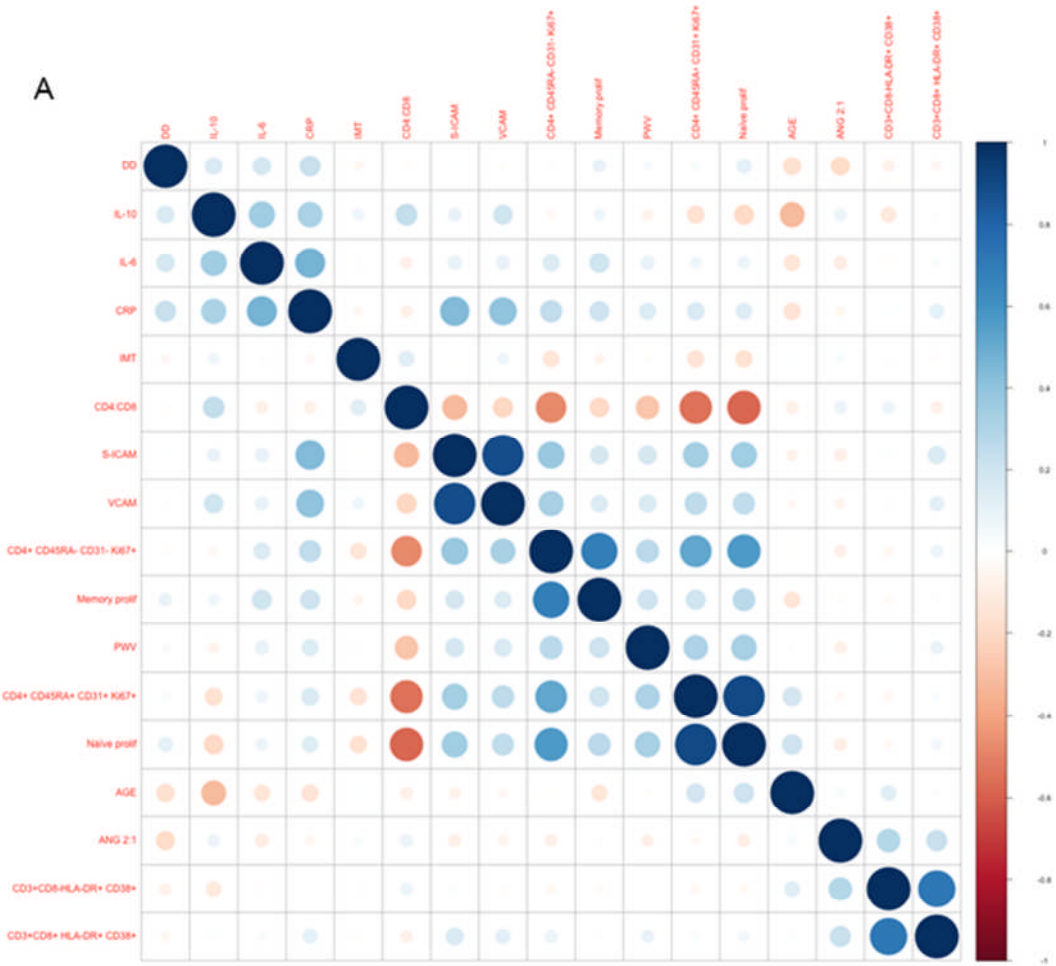
As illustrated in Figure 7-12, interestingly similar to the patterns seen in ART naïve and experienced children significant correlations exist between:

- the 2 markers of activation CD4+HLADR+CD38+ and CD8+HLADR+CD38+ (r=0.72)
- CD4+CD45RA-CD31-Ki67+ cells and the total proportion of proliferating CD4+ memory cells (r=0.68)
- CD4+CD45RA+CD31+Ki67+ and the total number of CD4+ naïve cells that were proliferating (r=0.89)
- No significant correlation between immunophenotyping data, IMT, PWV and age (r all ≤0.35)

Additionally some associations seen only in naïve children were also seen in the HIV uninfected controls:

- CD4+CD45RA-CD31-Ki67+ cells were positively correlated with CD4+CD45RA+CD31+Ki67+ (r=0.52) and the total proportion of CD4+ naïve proliferating cells (r=0.57)
- CD4:8 ratio was negatively associated with the total proportion of CD4+ naïve proliferating cells (r=-0.57)

Finally significant negative associations were observed between the CD4:8 ratio and CD4+CD45RA-CD31-Ki67+ / CD4+CD45RA+CD31+Ki67+ cells (r =-0.48/-0.54) in the HIV uninfected group only.


A
DD
IL-10
IL-6
CRP
IMT
CD4 CD8
S-ICAM
VCAM
CD4+ CD45RA- CD31- Ki67+
Memory prolif
PWV
CD4+ CD45RA+ CD31+ Ki67+
Naïve prolif
AGE
ANG 2:1
CD3+CD8-HLA-DR+ CD38+
CD3+CD8+ HLA-DR+ CD38+

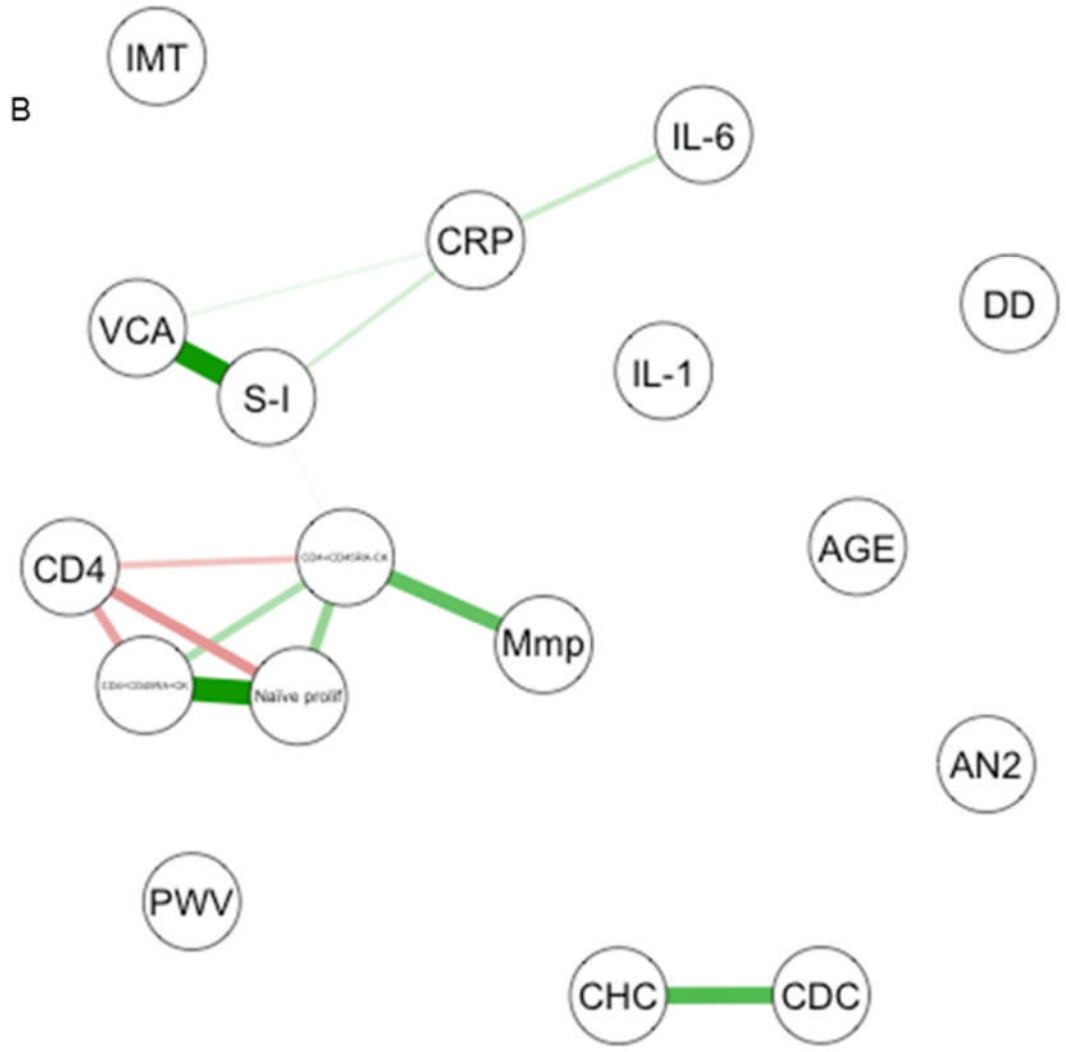


Figure 7-12. Relationship between immunophenotyping, biomarkers, viral load, age, IMT and PWV at baseline in HIV-uninfected children.

In the correlation matrix(A) the strength of positive relationships are demonstrated using blue scale and negative correlations on the red scale. In the nodal diagram (B) relationships with a spearman correlation of >0.35 are demonstrated, positive correlations shown in green and negative correlations in red.

AN2, = ANG2:1, ratio of angiopoietin-2:1 CD4, CD4:CD8, CDC, CD3+CD8-HLA DR+ CD38+, CHC, CD3+CD8+HLA DR+ CD38+, CRP, C reactive protein; DD, d-dimer; IL-10, interleukin-10; IL-6, interleukin-6; SI, IMT, intimal medial thickness; Mmp, proportion of memory cells that are proliferating; naïve prolif, proportion of naïve cells that are proliferating PWV, pulse wave velocity; sICAM, soluble intracellular adhesion molecule; VCA, soluble vascular cell adhesion molecule-1; VL, viral load;

7.5.6 Does duration of ART influence markers of activation and proliferation?

Looking just at the HIV-infected, ART experienced children no relationship between age at starting ART and markers of activation on CD4+ and CD8+ T-cells was seen (p=0.47/0.35). A significant inverse relationship between levels of both CD4+ and CD8 HLA DR+ CD38+ cells and duration on ART was demonstrated (p=0.03 / 0.01) - Figure 7-13. Comparing age at starting ART and duration on ART with the levels of proliferating CD4+ subpopulations in the HIV-infected, ART experienced children, no significant relationship was identified with age at starting ART (all p≥0.56), whereas the effect of duration on ART persisted.

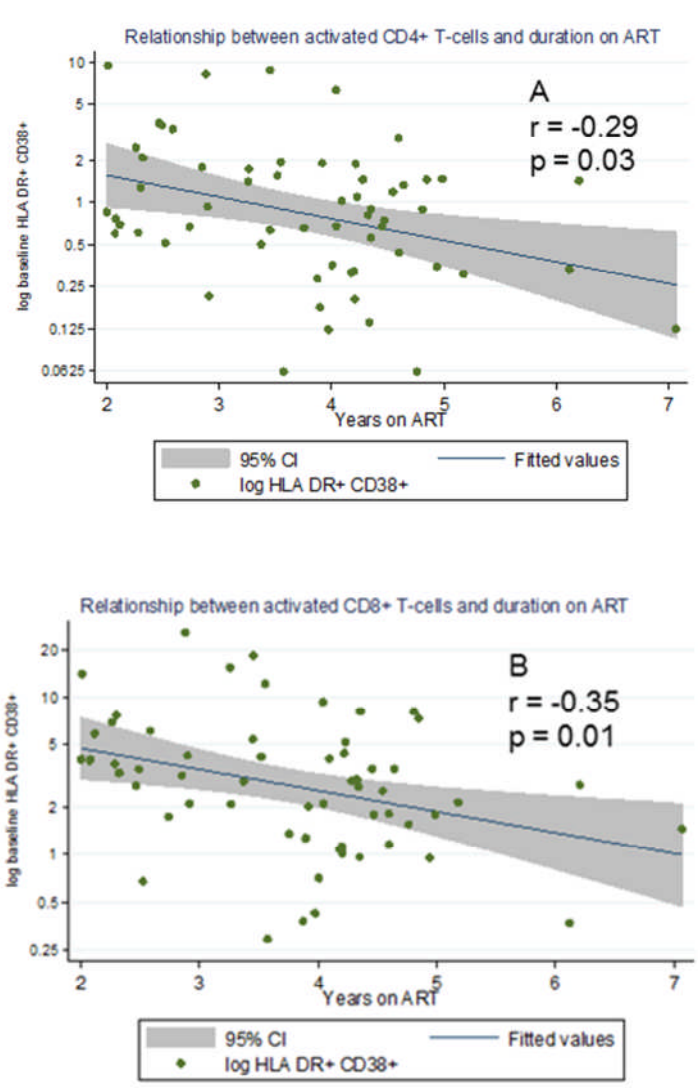


Figure 7-13. The relationship between duration of ART and markers of activation on CD4+ and CD8+ T-cells.

A significant relationship between markers of activation and duration on ART is demonstrated in both CD4+ and CD8+ T-cells.

7.5.7 Can T-cell activation or proliferation predict baseline markers of cardiovascular structure and arterial stiffness?

Not all children had both baseline IMT / PWV and activation / proliferation panels available. As summarised in Table 7-11 the following calculations were performed on the 60 – 84% of children with both measurements available.

Table 7-11. Summary of available cardiovascular, activation and proliferation results by group

	HIV+ ART naïve (group 1)	HIV- controls matched to group 1	HIV+ ART experienced (group 2)	HIV- controls matched to group 2
Total recruited	208	209	74	75
Baseline IMT (% total)	145 (70%)	167 (80%)	72 (97%)	67 (89%)
Baseline PWV (% total)	141 (68%)	155 (74%)	73 (99%)	75 (100%)
Baseline DR (% total)	177 (85%)	162 (78%)	60 (81%)	62 (83%)
Baseline Ki67 (% total)	187 (90%)	161 (77%)	60 (81%)	63 (84%)
Patients with both baseline IMT and activation (DR) panel (% total)	127 (61%)	135 (65%)	59 (80%)	56 (75%)
Patients with both baseline IMT and proliferation (Ki67) panel (% total)	129 (62%)	136 (65%)	59 (80%)	57 (76%)
Patients with both baseline PWV and (activation (DR) panel (% total)	124 (60%)	130 (62%)	60 (81%)	62 (83%)
Patients with both baseline PWV and proliferation (Ki67) panel (% total)	126 (61%)	129 (62%)	60 (81%)	63 (84%)

For IMT in HIV infected, ART naïve and controls 3 variables (CD4+CD45RA+CD31+, CD4+CD45RA+CD31-Ki67+ and CD4+CD45RA-CD31- added significant ($p \leq 0.03$) prognostic information to the previous model including total cholesterol, age, site plus case/control status (section 5.5.3) when included one at a time. Including these three together showed that only CD4+CD45RA+CD31+ and CD4+CD45RA+CD31-Ki67+ were independent predictors. The effects of age, total cholesterol, site and case/control were unchanged but IMT was independently higher in children with greater CD4+CD45RA+CD31+ and CD4+CD45RA+CD31-Ki67+ Table 7-12.

For IMT in HIV infected, ART experienced and controls 1 variable (CD4+CD45RA-CD31+Ki67+ added significant (p=0.01) prognostic information to the previous model including weight for age, age, site plus case/control status (section 5.5.3). Adding the effect of CD4+CD45RA-CD31+Ki67+ the effects of age, weight for age, site and case/control were unchanged but IMT was independently lower in children with a greater proportion of CD4+CD45RA-CD31+Ki67+ cells, Table 7 12.

Table 7-12. Final multivariable models for baseline IMT including the addition of immunophenotyping data.

	Estimate of impact on IMT (mm)	95% CI	p value
Naïve & controls			
Mean at reference category	+ 0.452	(+ 0.426 : + 0.478)	<0.001
Total cholesterol (per 1 mmol/L higher)	- 0.005	(-0.009 : - 0.001)	0.01
HIV-infected case vs uninfected control	+ 0.010	(+ 0.020 : + 0.004)	0.04
Site (UTH vs JCRC)	- 0.012	(+ 0.025 : + 0.001)	0.08
Age (per 1 year older)	- 0.001	(- 0.004 : + 0.001)	0.22
CD45RA+ CD31+	+ 0.0004	(+ 0.0001 : + 0.001)	0.01
CD45RA+ CD31+ Ki67+	+ 0.003	(+ 0.0004 : + 0.005)	0.02
Experienced & controls			
Mean at reference category	+ 0.480	(+ 0.445 : + 0.514)	<0.001
HIV-infected case vs uninfected control	+ 0.026	(+ 0.004 : + 0.048)	0.01
Weight-for-age in cases (per 1 unit higher)	+ 0.010	(+ 0.0004 : + 0.019)	0.04
Weight-for-age in controls (per 1 unit higher)	+ 0.002	(- 0.010 : + 0.006)	0.64
Site (UTH vs JCRC)	- 0.003	(-0.017 : + 0.012)	0.72
Age (per 1 year older)	+ 0.001	(- 0.003 : + 0.004)	0.80
CD45RA- CD31+ Ki67+	- 0.002	(-0.004 : 0.000)	0.01

Reference category = age 0 and total cholesterol 0. IMT, Intimal Medial Thickness; JCRC, Joint Clinical Research Centre, Kampala; UTH, University Teaching Hospital, Lusaka;

7.6 Longitudinal changes in markers of activation and proliferation

7.6.1 Activation

As summarised in Table 7-13 and illustrated in Figure 7-14 significant changes occurred in total lymphocytes, CD4+ and CD8+ activated (HLA DR+ CD38+) T-cells percentages and the ratio of CD4 to CD8 over 96 weeks in the children who were ART naïve at baseline. Lymphocytes and the CD4:CD8 ratio increased over the 96 weeks whilst the markers of activation fell (all p<0.001). The children who were ART experienced at baseline had a significant increase in the percentage of lymphocytes (p<0.001) whilst no significant change in the proportion of activated CD4+ and CD8+ T-cells were seen (p≥0.25). By 96 weeks absolute levels of activated CD4+ and CD8+ T-cells in HIV-infected naïve children (initiating ART) were similar to those in ART-experienced children and HIV-uninfected controls at baseline.

Table 7-13. Changes in markers of activation and CD4:CD8 ratio over 96 weeks by group.

	Group	Week 0			Week 48			Week 96			Difference between logweek 96 and log week 0
		No	Median	IQR	No	Median	IQR	No	Median	IQR	
Lymphocytes (% of total white cells)	1	177	26.6	(17.7 : 34.9)	161	31.6	(20.2 : 41.1)	141	37.1	(27.2 : 44.9)	**<0.001**
	2	60	31.1	(26.1 : 37.1)	61	36.7	(29.4 : 41.3)	63	40.5	(33.9 : 50.6)	**<0.001**
% of CD4+ expressing HLA DR+ CD38+	1	177	4.2	(1.9 : 8.0)	161	1.1	(0.5 : 2.2)	141	1.3	(0.8 : 2.4)	**<0.001**
	2	60	0.8	(0.4 : 1.6)	61	0.6	(0.3 : 1.0)	63	1.1	(0.6 : 2.0)	0.79
% CD8+ expressing HLA DR+ CD38+	1	177	12.1	(6.7 : 20.4)	161	3.3	(1.4 : 6.1)	141	3.6	(2.2 : 7.1)	**<0.001**
	2	60	2.9	(1.5 : 4.8)	61	2.7	(1.1 : 4.1)	63	2.9	(1.8 : 5.3)	0.91
CD4:CD8	1	177	0.4	(0.2 : 0.7)	161	1.0	(0.6 : 1.5)	141	1.1	(0.6 : 1.7)	**<0.001**
	2	60	0.8	(0.6 : 1.4)	61	1.1	(0.7 : 1.2)	63	0.9	(0.7 : 1.3)	0.25

Log10 values used to calculatechanges over time. Wilcoxon paired test to look at change over 96 weeks, Kruskal Wallis used to calculate difference between arms

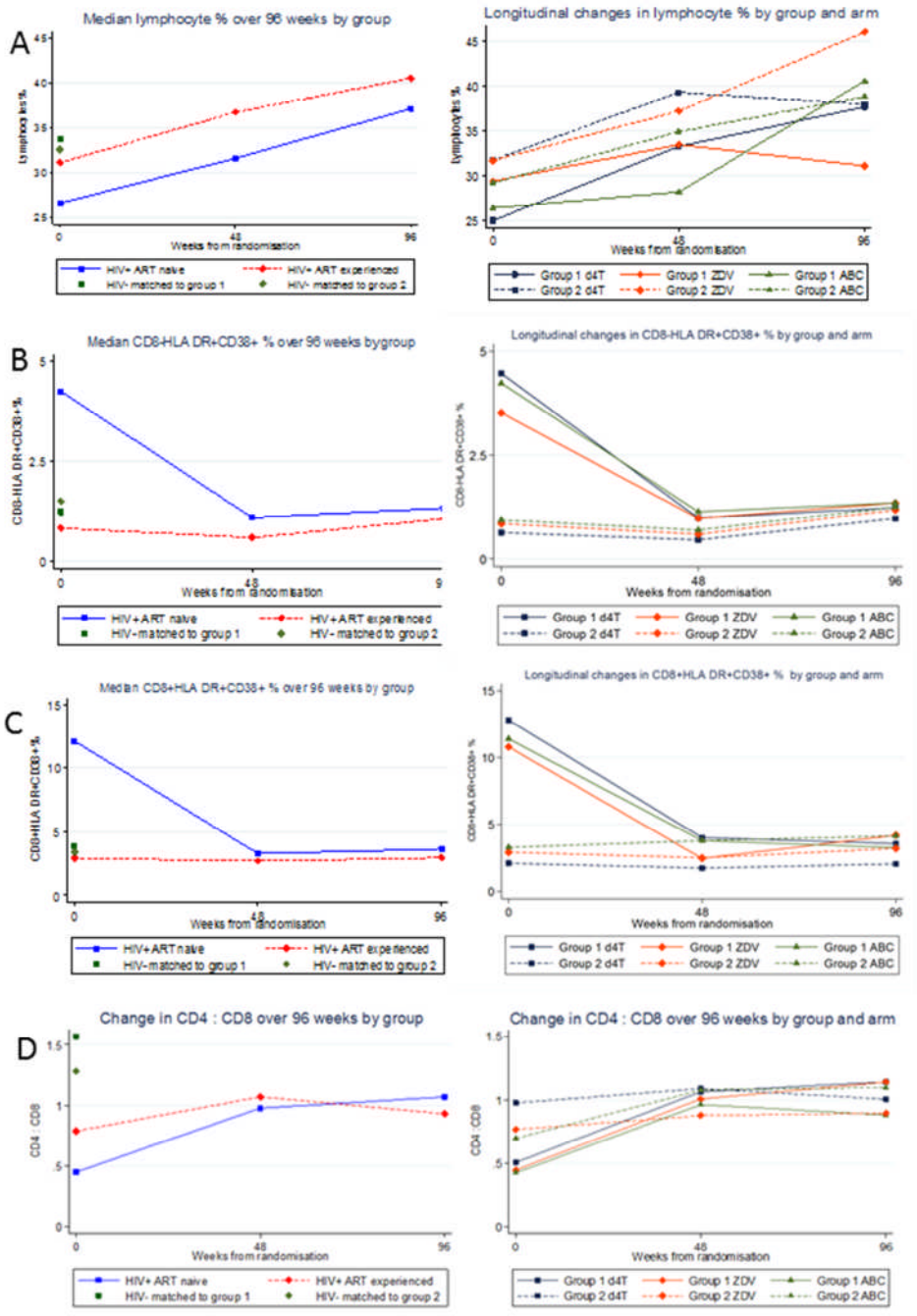


Figure 7-14. Changes in lymphocytes, markers of activation and CD4:CD8 ratio over 96 weeks by ART naïve vs experienced at enrolment and by randomised NRTI.

7.6.2 Proliferation

Results are summarised in Table 7-14 and illustrated in Figure 7-15 and 7-16.

Table 7-14. Changes in naïve and memory CD4+ T-cells and markers of proliferation over 96 weeks.

	Group	Week 0			Week 48			Week 96			Difference between logweek 96 and log week 0
		No	Median	IQR	No	Median	IQR	No	Median	IQR	
Markers of proliferation in CD4+ T-cells											
% CD4+ Ki67+	1	187	6.4	(3.9 9.2)	157	1.8	(1.1 3.3)	150	2.2	(1.2 3.9)	**<0.001**
	2	60	3.6	(2.7 4.7)	63	2.6	(1.7 3.9)	62	2.9	(1.8 3.9)	**<0.001**
Naïve cells											
% CD4+ CD45 RA+	1	187	57.9	(46.6 67.8)	157	68.7	(60.6 74.8)	150	69.6	(61.2 74.7)	**<0.001**
	2	60	59.0	(52.0 68.0)	63	63.9	(57.4 70.0)	62	64.4	(55.2 69.1)	**0.01**
% CD4+ CD45 RA+ Ki67+	1	187	3.4	(1.6 6.5)	157	1.2	(0.7 2.9)	150	1.3	(0.7 2.9)	**<0.001**
	2	60	2.3	(1.5 4.4)	63	2.4	(1.0 4.1)	62	3.2	(0.8 5.7)	0.14
% CD4+ CD45RA+ CD31+ (Q2)	1	187	48.4	(38.1 59.9)	157	59.1	(51.8 65.7)	150	59.8	(51.9 65.4)	**<0.001**
	2	60	51.2	(44.1 59.6)	63	56.0	(51.2 62.0)	62	53.6	(47.6 61.2)	0.07
% CD4+ CD45RA+ CD31+ Ki67+	1	187	1.4	(1.0 : 2.4)	157	0.8	(0.4 : 1.4)	150	0.6	(0.4 : 1.5)	**<0.001**
	2	60	1.2	(0.8 : 1.5)	63	1.3	(0.6 : 2.5)	62	1.6	(0.5 : 2.7)	0.82
% CD4+ CD45RA+ CD31- (Q3)	1	187	8.1	(4.6 11.8)	157	8.6	(6.0 11.7)	150	8.7	(6.7 12.2)	**<0.001**
	2	60	5.3	(3.6 8.0)	63	7.1	(5.1 9.5)	62	7.1	(4.7 12.4)	**0.003**
% CD4+ CD45RA+ CD31- Ki67+	1	187	1.6	(0.5 : 4.1)	157	0.6	(0.1 : 1.2)	150	0.6	(0.2 : 1.5)	**<0.001**
	2	60	1.1	(0.6 : 2.6)	63	0.8	(0.3 : 2.0)	62	1.1	(0.3 : 3.1)	0.68
Memory cells											
% CD4+ CD45 RA-	1	187	41.8	(32.2 50.4)	157	30.9	(25.2 38.2)	150	30.5	(25.3 38.1)	**<0.001**
	2	60	40.5	(31.6 47.4)	63	35.5	(30.0 42.6)	62	35.3	(30.7 43.8)	**0.003**
% CD4+ CD45 RA- Ki67+	1	187	23.2	(15.9 31.9)	157	6.9	(3.4 12.4)	150	9.1	(4.2 15.1)	**<0.001**
	2	60	12.7	(9.7 15.6)	63	9.8	(6.6 13.8)	62	10.3	(6.4 12.7)	**0.003**
% CD4+ CD45RA- CD31+ (Q1)	1	187	16.9	(11.6 22.6)	157	9.4	(6.8 13.7)	150	5.9	(3.7 11.7)	**<0.001**
	2	60	17.0	(9.9 22.6)	63	11.6	(6.7 17.4)	62	9.5	(5.3 19.6)	**<0.001**
%CD4+ CD45 RA- CD31+ Ki67+	1	187	11.9	(7.3 : 16.1)	157	3.7	(1.7 : 6.2)	150	4.4	(1.8 : 7.1)	**<0.001**
	2	60	5.6	(4.1 : 7.5)	63	4.8	(3.1 : 6.9)	62	4.5	(2.8 : 6.3)	**0.02**
% CD45RA- CD31-(Q4)	1	187	22.7	(13.3 . 33.4)	157	20.9	(15.1 27.2)	150	23.3	(17.7 28.6)	**0.01**
	2	60	23.2	(16.9 27.8)	63	23.9	(16.9 29.2)	62	23.2	(17.3 30.7)	0.79
%CD4+ CD45RA- CD31-	1	187	11.9	(7.2 : 16.6)	157	3.4	(1.3 : 6.7)	150	4.8	(1.9 : 8.1)	**<0.001**
	2	60	6.4	(5.0 : 8.2)	63	5.2	(2.9 : 6.4)	62	4.9	(2.9 : 7.1)	**0.01**

Log10 values used to calculate changes over time. Wilcoxon paired test to look at change over 96 weeks, Kruskal Wallis used to calculate difference between arms. Group 1: ART naïve at baseline Group 2: ART experienced at baseline

Total CD4+ T-cell proliferation

A significant decrease in the proportion of proliferating CD4+ T-cells was observed over 96 weeks in both children who were ART naïve and experienced at baseline ($p<0.001$) - Figure 7-15 A and B – with the decrease predominantly occurring by 48 weeks in both groups.

Total naïve cells

A significant increase in naïve CD4+ T-cells was observed over 96 weeks in both children who were ART naïve and ART experienced at baseline ($p\leq0.01$), with most of the increase by 48 weeks. In ART naïve children a significant reduction in the proportion of proliferating CD4+ T-cells was seen 48 weeks after starting ART ($p<0.001$), a decrease that was maintained until 96 weeks. In contrast, in ART experienced children a further 96 weeks of ART did not significantly affect the proportion of proliferating cells ($p=0.14$ compared to baseline) - Figure 7-15 C - F.

Naïve CD4+ CD31 subpopulations

A significant increase was seen in the CD45RA+ CD31+ subpopulation by 48 weeks after commencing ART in the ART naïve children, together with a significant reduction in the proportion of this population that were proliferating, changes that were maintained to 96 weeks ($p<0.001$ versus baseline). In contrast, there were no significant changes in the proportion of RTEs nor the level of proliferation within the ART experienced group following a further 96 weeks of ART ($p\geq0.07$) - Figure 7-16 A - D.

Within the CD31- subpopulation (central naïve cells) significant increases were seen in both ART naïve and ART experienced children after 96 weeks of ART ($p\leq0.003$). A significant fall in the proportion of proliferating central naïve cells was seen by week 96 in the ART naïve children ($p<0.001$) whilst no significant change in the proportion of proliferating central naïve cells was seen in the ART experienced group ($p=0.68$) - Figure 7-16 E - H. Data is also presented by NRTI randomisation arm which will be discussed in further detail in section 7.7.

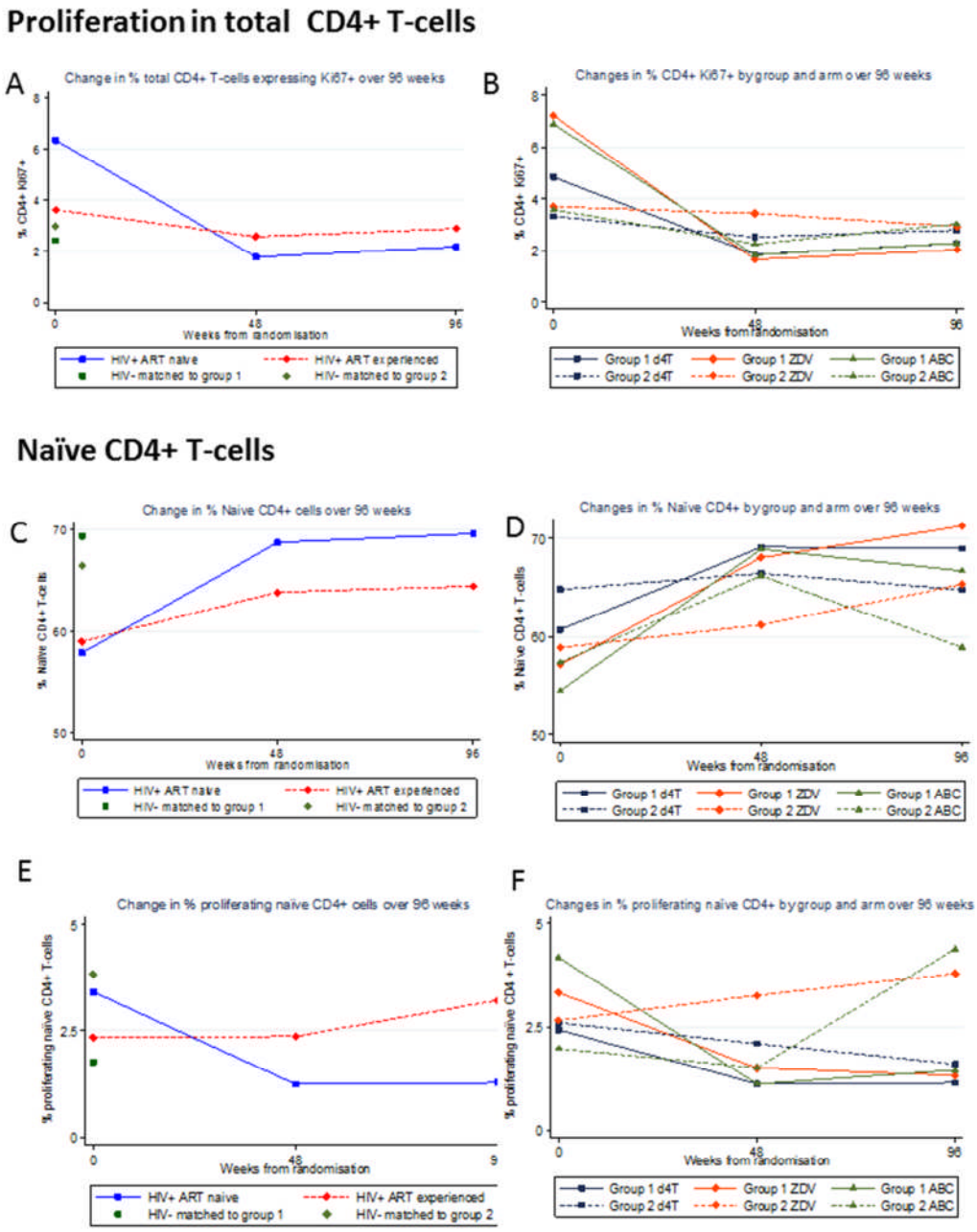


Figure 7-15. Changes in total proliferating CD4+ T-cells and proportions of naïve CD4+ cells over 96 weeks.

Effect of baseline group (ART naïve v ART experienced) demonstrated and effect of NRTI randomisation arm (d4T v ZDV v ABC).

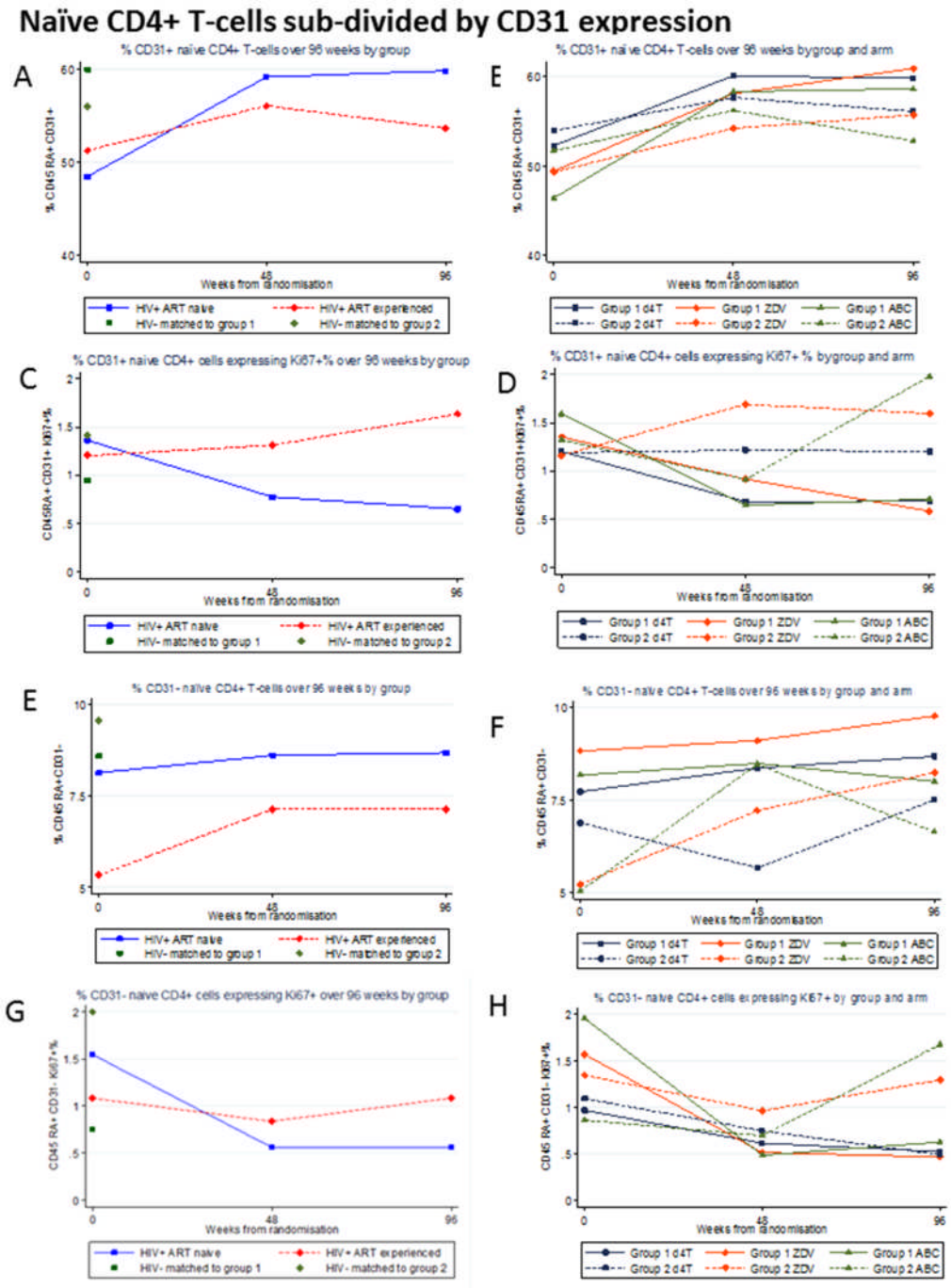


Figure 7-16. Changes proportions of naïve CD4+ T-cells subpopulations over 96 weeks

Effect of baseline group (ART naïve v ART experienced) demonstrated and effect of NRTI randomisation arm (d4T v ZDV v ABC).

Total memory CD4+ T-cells

Significant reductions in the proportion of total memory CD4+ T-cells and proliferating memory cells was demonstrated in both ART naïve and ART experienced children after 96 weeks on ART ($p \leq 0.02$) - Figure 7-17. Levels at 96 weeks were similar to the control groups.

Memory CD4+ CD31 subpopulations

A significant decrease in the total CD31+ memory cell population was seen over 96 weeks in both ART naïve and ART experienced children accompanied by a significant decrease in the proportion of this population that was proliferating ($p \leq 0.003$) - Figure 7-18 A - D.

In the CD31- memory population a significant increase was demonstrated by week 96 in the ART naïve group ($p=0.01$) whilst no significant difference was seen over 96 weeks in the ART experienced group ($p=0.79$). However, both ART naïve and ART experienced children had significant reductions in the proportion of CD31- memory T-cells that were proliferating over 96 weeks ($p \leq 0.01$) - Figure 7-18 E - H.

Memory CD4+ T-cells

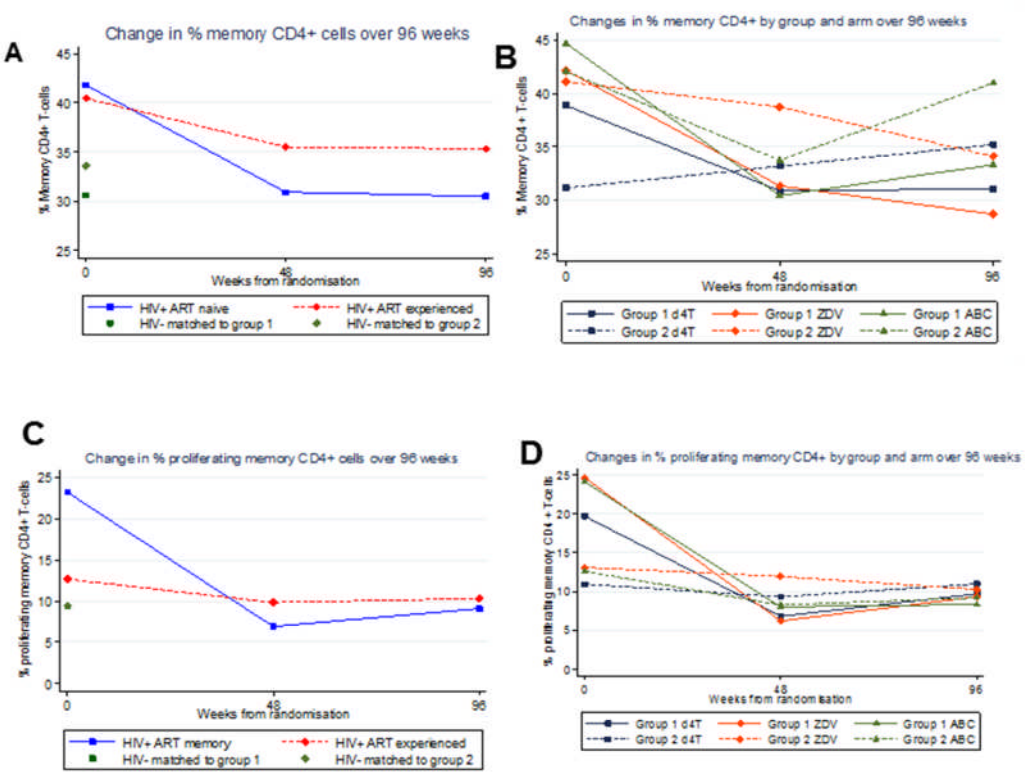


Figure 7-17. Change in total and proliferating memory CD4+ T-cells over 96 weeks

Effect of baseline group (ART naïve v ART experienced) demonstrated and effect of NRTI randomisation arm (d4T v ZDV v ABC).

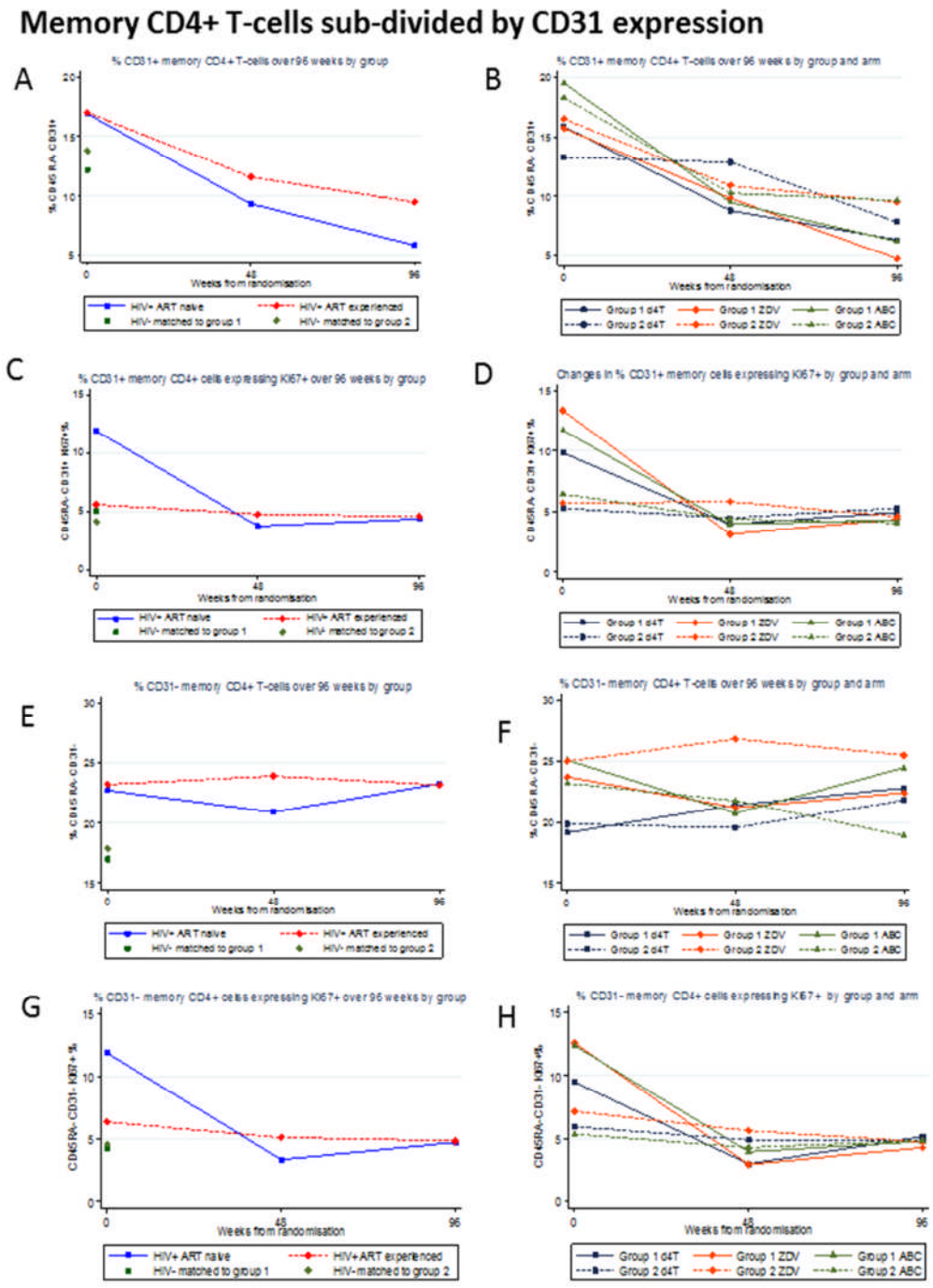


Figure 7-18. Changes in proportions of memory CD4+ T-cells subpopulations over 96 weeks

Effect of baseline group (ART naïve v ART experienced) demonstrated and effect of NRTI randomisation arm (d4T v ZDV v ABC).

7.7 Impact of NRTI regimen on changes in immunophenotyping data

Adjusted regression analysis was used to investigate the effect of randomized NRTI on mean change in the immunophenotyping markers over 48 and 96 weeks. The numbers of children overall and by group who had complete data at baseline and weeks 48 and 96 is summarised in Table 7-15.

Table 7-15. Summary of patients who had complete immunophenotyping data at baseline, week 48 and week 96.

		Activation panel (DR)	Proliferation panel (Ki67)
Overall Group 1 and 2	**Total**	**153**	**157**
	d4T arm	*47*	*51*
	ZDV arm	*54*	*55*
	ABC arm	*52*	*51*
ART naïve at baseline (group 1)	**Total**	**110**	**114**
	d4T arm	*35*	*38*
	ZDV arm	*35*	*37*
	ABC arm	*40*	*39*
ART experienced at baseline (group 2)	**Total**	**43**	**43**
	d4T arm	*12*	*13*
	ZDV arm	*19*	*18*
	ABC arm	*12*	*12*

ABC, abacavir; d4T, stavudine; ZDV, zidovudine;

As overall changes in each IPT subpopulation were broadly similar in naïve and experienced children (figures 7-12 to 7-15) I pooled both groups to gain the greatest power to detect any differences between randomized NRTI. I then checked that this pooled analysis had not masked detrimental effects of randomized NRTI according to whether the child was naïve or experienced by testing for significant interactions between randomized NRTI and naïve / experienced strata. All models included the baseline IPT variable as a factor to adjust for regression to the mean effects.

Of 26 IPT markers compared at two time-points (52 models), I found only one interaction p-value <0.05 (Table 7-16). Therefore there is no evidence that pooling naïve and experienced groups is masking genuine differences between randomized NRTIs.

Considering these pooled (main effects) models, if there were no differences between randomized NRTI groups I would expect 26 IPT markers explored at 2 time points to have 26*2*0.05= 2.6 global tests of differences between NRTI to have a p<0.05 and 26*2*0.1=5.2 tests to have a p<0.1 just by chance.

In fact three global tests were p<0.05 (Table 7-16); the percentage of CD4+CD45RA+CD31+Ki67+ cells at 48 and 96 weeks (global p=0.05/0.01), and the percentage CD4+CD45RA-CD31+Ki67+ cells at 48 weeks (global p=0.04). There was no consistent evidence of a difference between randomized groups in change in any of the immunophenotyping markers from baseline to week 48 or 96 adjusting for site, age and baseline immunophenotyping marker. Given the multiple tests performed it is likely that the results are a reflection of multiple testing rather than genuine differences.

Table 7-16. Results (p-values) of adjusted regression analysis to investigate effects of randomised NRTI on change in immunophenotyping markers over 48 and 96 weeks.

Randomised NRTI comparisons from a main effects model: interaction model also fitted to check for effect modification by ART naïve vs experienced group. In the interaction model columns, p-values for modification by ZDV (vs d4T) and ABC (vs d4T) are shown

	Week 48				Week 96			
	Global test	d4T v ZDV	d4T v ABC	ZDV v ABC	Global test	d4T v ZDV	d4T v ABC	ZDV v ABC
General								
Lymphocytes	0.30	0.96	0.19	0.17	0.57	0.29	0.62	0.55
% CD3+	0.72	0.44	0.84	0.57	0.43	0.26	0.98	0.26
% CD4+	0.95	0.75	0.82	0.93	0.84	0.69	0.89	0.57
% CD3+ CD8+	0.75	0.45	0.80	0.62	0.99	0.91	0.97	0.94
CD4:CD8	0.69	0.73	0.62	0.40	0.98	0.91	0.94	0.85
Markers of activation								
CD4								
% CD4+ DR- CD38+ (Q1)	0.36	0.42	0.54	0.15	0.19	0.71	0.46	0.96
% CD4+ DR+ CD38+ (Q2)	0.63	0.35	0.52	0.77	0.92	0.75	0.98	0.72
% CD4+ DR+ CD38- (Q3)	0.07	0.18	0.36	**0.02**	0.26	0.43	0.10	0.38
% CD4+ DR- CD38- (Q4)	0.67	0.77	0.57	0.38	0.47	0.85	0.35	0.25
CD8								
% CD8+ DR- CD38+ (Q1)	0.68	0.83	0.40	0.53	0.21	0.11	0.15	0.86
% CD8+ DR+ CD38+ (Q2)	0.08	**0.05**	0.91	0.06	0.99	0.90	1.00	0.90
% CD8+ DR+ CD38- (Q3)	0.89	0.97	0.66	0.69	0.24	0.23	0.10	0.65
% CD8+ DR- CD38- (Q4)	0.65	0.41	0.96	0.44	0.50	0.95	0.33	0.29
Markers of proliferation in CD4+ T-cells								
% CD4+ Ki67+	0.07	**0.03**	0.09	0.63	0.15	0.06	0.49	0.22
Naïve cells								
% CD45 RA+	0.10	0.83	0.08	**0.05**	0.77	0.55	0.97	0.52
% CD45 RA+ Ki67+	0.36	0.47	0.50	0.15	0.42	0.32	0.84	0.22
% CD45RA+ CD31+ (Q2)	0.12	0.92	0.09	0.06	0.89	0.63	0.80	0.81
% CD45RA+ CD31+ Ki67+	**0.05**	0.09	0.50	**0.02**	**0.01**	0.16	0.14	**0.004**
% CD45RA+ CD31- (Q3)	0.58	0.91	0.41	0.34	0.84	0.66	0.90	0.57
% CD45RA+ CD31- Ki67+	0.86	0.79	0.58	0.76	0.51	0.25	0.46	0.67
Memory cells								
% CD45 RA-	0.13	0.47	**0.05**	0.19	**0.10**	0.08	0.88	**0.05**
% CD45 RA- Ki67+	**0.04**	**0.01**	0.41	0.09	0.19	0.08	0.62	0.2
% CD45RA- CD31+ (Q1)	0.33	0.83	0.25	0.17	0.68	0.99	0.46	0.44
% CD45RA- CD31+ Ki67+	0.07	**0.02**	0.12	0.48	0.37	0.16	0.45	0.51
% CD45RA- CD31-(Q4)	0.75	0.46	0.79	0.64	0.22	0.16	0.86	0.11
% CD45RA- CD31- Ki67+	0.06	**0.02**	0.46	0.11	0.16	0.09	0.88	0.12

7.8 Discussion

7.8.1 Activation

I have demonstrated, as others have previously found, a reduction in the proportion of activated T-cells expressing CD4+ HLA DR+ CD38+ and CD8+ HLA DR+ CD38+ with the use of ART in HIV-infected children [463-465]. I did not find any significant correlation between these as markers of activation and viral load, age, CD4 count, CD4 percentage or CD4 z score, this is in contrast with the findings of a smaller Spanish paediatric study that found levels of HLA DR CD38 correlated with viraemia [439]. Whilst no significant relationship between age at baseline or age at starting ART existed, duration of ART did prove to be inversely related to levels of activation. The significance of increased levels of activation were described in a study of Ugandan HIV-infected adults in whom higher levels of T-cell activation were significantly associated with increased mortality [455] conversely HIV viral load rather than degree of activation was found to be a stronger predictor of disease progression in a different Ugandan adult cohort [466].

7.8.2 Relationship between activation and proliferation

When all HIV infected and uninfected children at baseline were looked at a significant relationship between increasing activation (CD4+ and CD8+ HLA DR+ CD38+) and higher levels of proliferating CD4+ CD45RA- CD31+ and CD4+ CD45RA- CD31- T-cells was demonstrated. This was also seen when the ART naïve children were looked at separately but no significant relationship was seen in the smaller group of virologically suppressed ART experienced or in the HIV-uninfected children. The PENTA 11 study, examined the immunological and virological consequences of planned treatment interruption (PTI). Findings of PENTA 11 showed that in the PTI arm there was a rapid fall in the total CD4 cell in the first 12 weeks off ART, with decreases in both naïve and memory cells. However, the proportion of CD45RA+ and CD45RO+ CD4 cells remained constant in both the PTI and continuous treatment arm. The increase in CD8 cells in the first 12 weeks off ART in the PTI group was predominantly due to increases in RO+

expressing cells. PTI was associated with a rapid and sustained increase in CD4 cells expressing Ki67 and HLA-DR [318].

7.8.3 Changes in CD4 subpopulations

Throughout this thesis naïve cells have been defined by CD45RA expression; this will include CD45RA+ CD45RO- (truly naïve cells) and a very small proportion of primed CD45RA+ CD45RO+ cells. Work by Sefe (thesis 2012 unpublished) showed that >99% of CD45RA+ cells expressed both CD27 and CCR7, markers of naïve cells, suggesting that in a paediatric population the proportion of CD45RA+RO+ is very small. Memory cells have been defined by the lack of CD45RA expression; this will include both CD45RA-CD45RO+ and CD45RA- CD45RO- cells – the phenotype of the later in unknown and may be a population of apoptotic cells derived from memory cells [467] or contamination as the process to lyse HIV-infected red blood cells can be tricky as the cells are notoriously sticky.

CD4+ T-cells fall when destruction of CD4+ cells overcomes compensatory mechanisms that include proliferation of memory cells, increased naïve cell production and proliferation. At birth naïve cells predominate, levels fall throughout childhood accompanied by an increase in memory cells. In HIV a decrease in the naïve pool is seen with advancing immunosuppression.

With advanced HIV there is an increase in memory cells, which are a rapidly replicating population with shorter half-lives and telomeres compared to naïve cells, and are more susceptible to apoptosis and infection by HIV. I have shown that significantly increased numbers of memory cells are seen in both ART naïve and experienced children with both CD31+ and CD31- sub populations being significantly higher in HIV-infected children compared to controls. I have confirmed previous findings that in ART naïve children there is evidence that compensatory mechanisms continue to operate as evidenced by increased Ki67 expression on CD45RA- (memory) cells with declining CD4 count [318].

In adults commencing ART is associated with a redistribution of memory cells followed later by an increase in naïve cell production [319] whilst immune reconstitution of CD4+ T cells in HIV-infected children following the commencement

of ART follows a different pattern characterized by an early and sustained increase in naïve cells [47, 48, 465].

7.8.4 Recent thymic emigrants

CD45RA+ CD31+ T-cells are often referred to as recent thymic emigrants (RTE) [468, 469]. Similar to other findings I have shown that the proportion of RTEs decreased with age in HIV uninfected individuals and HIV-infected, ART naïve children [470]. I have also shown a significantly lower proportion of recent thymic emigrants in ART naïve children compared to their uninfected controls but the RTEs have higher levels of proliferation. HIV-infected children stable on ART have similar proportions of RTEs to their control group complementary to findings by Vrisekoop et al who demonstrated after 4 years on ART proportions of RTEs were similar between HIV-infected and uninfected controls [471]. I have shown that absolute CD4 counts are positively associated with a higher proportion of RTEs suggesting it is important to maintain naïve cell production to maintain CD4. Data from the ARROW study has shown that the proportion of naïve CD31+ cells prior to commencing ART is an important indicator of eventual immune recovery [472]. RTEs by virtue of their telomere length and telomerase activity are believed to have a very limited replicative ability [473] which is supported by my findings that only a very small proportion of RTE are actively proliferating. In healthy individuals <5% cells express Ki67.

The thymus is crucial in immune recovery and determining the ratio of naïve to memory cells. Thymic volume in HIV infected children is positively correlated with naïve CD4+ T-cells and inversely correlated with viral load [474]. Age adjusted thymic output is reduced in HIV-infected children; early ART can maintain thymic output and a longer duration on ART is associated with increased thymic output in children [475]. Higher thymic output is important for the reconstitution of naïve T-cells [476] however severe T-cell depletion at ART initiation in HIV infected children limits the ability for immune recovery [17, 477, 478].

7.8.5 Central naïve cells

CD31- naïve cells represent a population with lower TREC content in which CD31 expression is decreased in response to homeostatic expansion [470]. A model has been proposed of CD4 homeostasis whereby RTE in the periphery respond to antigen, proliferate and differentiate into central naïve cells (CD45RA+CD31-) to compensate for falling thymic output [470]. I have shown that the proportions of these CD45RA+ CD31- cells are significantly lower in HIV-infected ART experienced children compared to their control groups. A trend towards lower levels in ART naïve children compared to controls was seen but was not significant. Less work has focused upon this sub population of T-cells; differences in adult populations are subtle, are unaffected by age and it appears the proportion remains fairly constant. They may have a suppressor role further contributing to the immune dysregulation in HIV [479].

7.8.6 Vascular structure and arterial stiffness

As presented in section 7.5.7 higher levels of 2 variables (CD4+ CD45RA+CD31+ and CD4+ CD45RA+ CD31-Ki67+) in HIV infected ART naïve and HIV uninfected controls were independent predictors of an increase in IMT. In HIV infected, ART experienced higher proportions of CD4+ CD45RA-CD31+Ki67+ were associated with a thinner IMT. Previously published findings have shown mixed results, in one study in both ART naïve and experienced HIV-infected women higher T-cell activation levels significantly predicted carotid artery stiffness as measured by ultrasound assessment of atrial distension [480]. However no significant relationship between markers of immune activation and changes in IMT have been seen in other studies [245, 450, 451].

7.9 Conclusions and future work

Considerable heterogeneity exists within the immunophenotyping results; some ART naïve children are coping well before the introduction of ART with high levels of naïve cells and low levels of activation and proliferation, the opposite is true in some virologically suppressed ART experienced children. Understanding these complex relationships in more depth would be fascinating although fitting multiple regression models to each marker of activation and proliferation individually would be extremely

time consuming. A more efficient approach would be to use dimension reduction techniques such as principal component analysis (PCA) and clustering to identify underlying groups of children in the data, then try to characterise each group of children in terms of other markers.

Chapter 8 Discussion

8.1 How this study has contributed to the understanding of paediatric HIV?

The key aims of this thesis were:

1. To determine the influence of HIV infection on vascular phenotype by comparing HIV infected ART naïve children with HIV uninfected African controls

2. To determine the effects of ART on vascular phenotype by comparing children stable on ART to ART naïve about to start treatment and monitor changes in arterial stiffness over time.

3. To gain insight into the potential mechanisms operating to mediate vascular dysfunction

This study was conceived at a time when a large proportion of HIV infected children were not receiving ART, the decision to start ART after the 1st year of life was based upon immunological and clinical criteria. Since the publication of the START study results the World Health Organisation have released updated guidelines recommending all HIV-infected infants, children and adults commence lifelong ART as soon as possible following diagnosis [46, 481]. As such children will commence lifelong ART as soon as possible following diagnosis. For the majority of HIV infected children, where effective ART is available and taken, survival into adolescence and adulthood should be the norm however optimising effective ART combinations to minimise toxicities remains a priority. The main findings of CHAPAS-3 have shown that the three NRTI regimens studied were equally successful in terms of virological suppression, CD4 cell recovery with low levels of toxicity. The cardiovascular sub study results have added to these findings by showing that the three treatment arms were equally effective in improving markers of vascular structure/ arterial stiffness, biomarkers and immunophenotyping.

In chapter 5 I have addressed aims 1 and 2 by showing that even at a mean age of 2.9 years adverse changes are demonstrated in IMT and PWV compared to age

matched HIV-uninfected children. The benefits of ART are demonstrated with the ART experienced children in whom no significant difference in IMT and PWV was demonstrated compared to their HIV-uninfected controls. Duration of ART was demonstrated to be important in ART experienced children, who having been on ART for a mean of 3.7 years, following a further 96 weeks of ART had significant improvements in IMT. For PWV improvements were seen in ART naïve children commencing ART but an increase in PWV was demonstrated in ART experienced children. Unfortunately to date no data on the normal progression of PWV in healthy African children and adolescents are available and the significant increase in PWV over 96 weeks may be an expected age related finding regardless of HIV status.

Aim 3 was addressed in chapters 6 and 7. Originally the biomarker panels were set up a priori to cover 3 mechanisms of damage; inflammation, disordered thrombogenesis and cardiovascular injury/repair. All biomarkers studied were abnormal in ART naïve compared to controls and the majority of biomarkers in ART experienced were not restored to the levels measured in HIV uninfected controls. Whilst vascular function appears to be improving the biomarker results were not as straight forward. Interestingly, the biomarkers did not cluster as originally predicted and the interplay between viral replication, chronic HIV associated inflammation and the cumulative adverse effects of ART was complex. The biomarker and immunophenotyping work has provided rich data on normal ranges in HIV uninfected healthy African children upon which future work can develop.

An aim of identifying biomarkers that could be useful in predicting future atherosclerotic risk has not resulted in any clear biomarker changes or patterns that are predictive of measureable changes in either IMT or PWV, in contrast to other paediatric studies that have demonstrated a relationship between higher CRP and adverse changes in IMT [442]. Biomarkers fluctuate within an individual so it is difficult to use them to predict an individual's risk.

With very early diagnosis HIV-infected babies treated within a few days of birth have been shown to have a very small pool of integrated viruses [50], have an impressive capacity to regenerate their immunological repertoire [482, 483] and high proportions of relatively HIV resistant naïve T-cells [484]. This population of children offers an opportunity to investigate the potential efficacy of immune based therapies

and possible treatment interruption strategies. Understanding the dynamics of biomarker changes is one way in which these could potentially be utilized in guiding which seronegative HIV infected children could undergo treatment interruption strategies [485].

In chapter 4 I described the methods used to set up an African study of young children in resource limited settings. This is the largest study within a population with a high HIV burden and limited ART options. No studies had previously been published that included a sizeable proportion of children under the age of 5 years due to the belief that young infants and children would either be unable to tolerate the measurement of IMT and PWV or that anatomically their necks were too short. This study has shown that both are possible within this age group.

8.2 Strengths and limitations of the study

This was a prospective study with a significant and carefully matched control group that helped to ascertain the impact of HIV infection against a background risk of confounders such as for example co-infections and malnutrition. Studying vertically infected children allowed accurate dating of the time of infection. Within the population studied no child was obese; obesity has independent effects on markers of inflammation [438]. The confounding impact of different ART regimens has been limited by studying children on a limited number of ART regimens and with no use of PIs, which have been associated with metabolic derangement [486].

This is the first study to provide data from an African setting. Comparisons with Northern America and Europe studies must be interpreted with caution given the heterogeneous array of ART prescribed, differing prevalence of obesity and also the increased risk of vitamin D deficiency that may affect levels of immune activation within American / European cohorts [487]. Co-infections such as CMV and TB are commoner in an African setting and clear relationships between CMV infection and abnormalities in markers of inflammation are described [488].

Whilst this study has added to the understanding of IMT and PWV changes in HIV infected children compared to HIV uninfected children, deficiencies in our knowledge persist such as the natural progression of IMT / PWV changes in HIV infected ART

naïve children who do not commence ART. Given the current WHO guidelines all HIV infected children should be commenced immediately on ART, this question is unlikely to ever be answered. The control group provided important information and normative data but due to the difficulty recruiting controls, the number of staff available for repeat visits and financial limitations, the controls were only assessed at a single time point. The parameters measured had not been studied in this or similar populations previously so the data was used to devise population norms. It is of regret that is was not possible to collect longitudinal cardiovascular data to start to understand the natural progression of IMT and PWV in healthy HIV uninfected African controls.

8.3 Recommendations for future research

The findings presented within this thesis support the current WHO guidelines, to treat all HIV infected children early regardless of immunological or clinical status in that early and persistent treatment does make biological sense. As summarised in a recently published personal view, children have a greater risk of morbidity and mortality yet the greatest potential for recovery with early and prolonged ART [485]. Longer term follow up is needed with the growing cohort of HIV infected children who receive very early and prolonged ART however the normal changes in IMT, PWV and biomarkers in healthy HIV non infected children over time must be understood in order for results to be put into context.

In chapter 5 a surprising result that an increase in total cholesterol was associated with thinner (healthier) IMT was presented. Initially this seems counterintuitive however increasing evidence is emerging of the complex underlying changes in lipids in HIV infected adults and to lesser extent children. It has long been known that HIV infection causes an early decrease in HDL cholesterol, an elevation of triglycerides with a delayed decrease in LDL-C later in infection [489, 490]. However there may be more subtle changes underlying. HDL dysfunction may represent a novel mechanism linking inflammation with progression of atheroma; recent findings that even minor alterations in systemic inflammation can impair the endothelial protective effects of HDL [340]. Functionally defective high density lipoproteins (HDL) are related to heightened T cell activation in vertically HIV-infected

adolescents [491]. Published work showing the addition of statins on overall cardiovascular risk show mixed results to data [92, 492] and future trials will evaluate other interventions that effect lipids. Lipoprotein-associated phospholipase A2 (Lp-PLA2), a recently described marker of inflammation, oxidizes phospholipids on LDL in the arterial intima to produce oxidized free fatty acids and lysophosphatidylcholine which stimulate key steps in the atherosclerotic process. Early work has suggested that this may be of promise as a biomarker of CVD risk [493].

During this research abnormalities in other biomarkers such as the monocyte activation marker, sCD163 and the kynurenine-to-tryptophan (KT) ratio have been linked with increased cardiovascular risk in adults [316, 494]; no paediatric data exists to date. CMV and other co-infections are implicated in immune activation, increased cardiovascular disease and progression of HIV infection [495, 496]. Microbial translocation may be a key factor and has been implicated as part of the immunopathology underlying HIV disease progression and cardiovascular risk [497, 498]. Further work using CHAPAS-3 samples will focus on microbial translocation in more detail. What is not known is whether identifying and treating inflammation and immune dysfunction will decrease morbidity and mortality. The use of anti-inflammatory agents such as histone deacetylase inhibitors in HIV infected patients has shown that inflammatory markers can be reduced; this opens the potential for trials of therapeutic interventions for high risk patients with persistently high levels of inflammation [499].

8.4 Conclusions, key findings and implications for practice

Without ART, detrimental changes in vascular structure and arterial stiffness can be seen from a very young age but are in part reversible with ART. Biomarker data hints at aetiology and provides additional evidence that early ART is beneficial. Results are in accordance with current WHO guidelines that ART is life saving and children need to be started early on first line ART. Optimising first line ART is a careful consideration between cost, available formulations and adverse effects. CHAPAS 3 and the cardiovascular sub study have shown that there are no

significant differences in terms of toxicity or virological suppression between three first line NRTI options.

Ongoing work needs to focus on cardiovascular disease in vertically infected adolescents as they enter adulthood. In the meantime the medical care of HIV-infected children must recognise that they remain at increased cardiovascular risk; counselling over the need to remain active, avoid obesity, not to smoke and be screened for renal complications, hypertension and subclinical cardiovascular damage should be considered standard of care.

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Appendices

Appendix I: CHAPAS 3 cardiovascular clinical report forms

Consent form

Form B: Contact details

Form 1: Screening

Form 3: Enrolment

Form 4: Lipodystrophy

Form 17: Socioeconomic questionnaire

Form 19: Cardiovascular sub-study

Form 7: Lab request

Form 11: Haematology

Form 12: Biochemistry

Form 13: Lymphocyte subsets

Form 14: Sample Storage

Appendix I: Clinical report forms.

CHAPAS-3

CHAPAS-3 **CONTROL STUDY CONSENT (HIV UNINFECTED)**

DOB D D M M M Y Y Y Y | Visit date D D M M M 2 0 1 Y

Child's Initials | Male ○ Female ○ | Hospital/clinic number

CHAPAS-3 cardiovascular substudy CONSENT for PARENTS/GUARDIANS and HIV uninfected CHILDREN

Please initial (or mark) box if you agree

1. I/My child have read and understood the information sheet on the CHAPAS-3 cardiovascular substudy. I understand the benefits and disadvantages of my child participating in this study. The details of this study have been explained to me by Dr ..... and my questions have been answered satisfactorily.	
2. I/My child know that my child can be withdrawn from the study at any time, without giving a reason and without it affecting his/her normal care and management.	
3. I/My child understand that information may be reviewed by properly authorised individuals as part of the study and that such information will be treated as strictly confidential.	
4. I/my child agree to allow a blood sample to be taken from my child, stored for testing later and for possible use in further studies on HIV. I understand that these results and samples will not be identified by either my or my child's name and that we may not be given the results of any tests performed on stored samples.	

Carer's signature (or thumbprint)	Print name	Date
		D D M M M 2 0 1 Y
Child's signature (or thumbprint) *where appropriate*	Print name	Date
		D D M M M 2 0 1 Y
Witness's signature (if carer cannot read)	Print name	Date
		D D M M M 2 0 1 Y
Doctor's signature	Print name	Date
		D D M M M 2 0 1 Y

IMPORTANT: original should be filed in trial file, a copy filed in the child's clinic notes, and one given to the family together with a copy of the information sheet(s) used.

CHAPAS-3

CHAPAS-3 TRIAL **FORM B – CONTACT DETAILS - CONTROLS**

Trial No | | | | | | |

Male ○ Female ○

Date of Birth D D M M M Y Y Y Y

Form Date D D M M M 2 0 1 Y

Child's Initials | |

Clinic number | | | | | | | |

Centre: UTH ○ JCRC ○

CONTACT DETAILS

1. **Full name of child** | | | | | | | | | | | | | | | | | | | | | | | | | |

(a) Home village _____

(b) Address / locality* _____

_____

_____

(c) Phone number | | | | | | | | | | | | | | | | | | | | | | | | | |

(d) Is this a personal phone number? N/A ☐ Yes ☐ No ☐ If no, specify: _____

If yes: (i) Are you happy for us to phone you if necessary? Yes ☐ No ☐

(ii) If you are not available, are you happy for us to leave a message?
On answerphone/voicemail for the number above? Yes ☐ No ☐
With someone who answers these numbers? Yes ☐ No ☐

2. **Name of 1st carer** | | | | | | | | | | | | | | | | | | | | | | | | | |

Relationship to the child?

Mother ..... ☐ Father ..... ☐ Brother ..... ☐ Sister ..... ☐
Aunt ..... ☐ Uncle ..... ☐ Cousin ..... ☐ Grandmother (maternal) ☐
Grandmother (paternal).. ☐ Grandfather (maternal) . ☐ Grandfather (paternal) .. ☐ Friend ..... ☐
Neighbour ..... ☐ Other (specify) ☐ _____

(a) Home village _____

(b) Address / locality* (If different to child) _____

_____

_____

* Draw map on next page if necessary

(c) Phone number | | | | | | | | | | | | | | | | | | | | | | | | | |

(d) Is this a personal phone number? N/A ☐ Yes ☐ No ☐ If no, specify: _____

If yes: (i) Are you happy for us to phone you if necessary? Yes ☐ No ☐

(ii) If you are not available, are you happy for us to leave a message?
On answerphone/voicemail for the number above? Yes ☐ No ☐
With someone who answers these numbers? Yes ☐ No ☐

Completed by (signature)	Print name	Date
		D D M M M 2 0 1 Y

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CHAPAS-3

CHAPAS-3 STUDY **CONTROLS**

Study No | Male ○ Female ○ | Date of Birth D D M M M Y Y Y Y

Visit Date D D M M M 2 0 1 Y | Child's Initials | Clinic number

Centre: UTH ○ JCRC ○

CHAPAS-3 control study patient information sheet provided and screening consent obtained? Yes ☐ No ☐
If no, do not continue.

Please note that form and question numbers follow the main CHAPAS 3 trial forms, however the parts that are not relevant to the control study have been deleted.

FORM 1 - SCREENING

ELIGIBILITY CRITERIA at SCREENING

9. Eligible (All must be Yes)		**Yes**	**No**
b)	Aged 1 month to 13 years	☐	☐
c)	Weighing ≥3kg and <30kg?	☐	☐
d)	HIV-1 uninfected, determined by 1 negative antibody test or if < 18 months 1 positive antibody result and 1 negative PCR	☐	☐
h)	**No** acute infections and **not** on anti-epileptics or insulin?	☐	☐
i)	Girls: **Not** pregnant or breastfeeding?	☐	☐

If any answers in shaded box do not continue

FORM 3 – ENROLMENT

HISTORY and PHYSICAL EXAMINATION TODAY

Height ☐☐☐.☐ cms Weight ☐☐.☐ kgs Temperature ☐☐.☐ °C Head circ (if < 5 yrs) ☐☐.☐ cms

1. Record clinical examination findings below:

	Normal	Abnormal	If Abnormal, give details
Eyes	☐	☐	
Ear, nose, mouth and throat	☐	☐	
Clinical anaemia	☐	☐	
Chest/cardiovascular	☐	☐	
Liver	☐	☐	
Spleen	☐	☐	
Lymph nodes	☐	☐	
Skin, hair and nails	☐	☐	
Musculo-skeletal system	☐	☐	
Nervous system	☐	☐	
Genital examination (if indicated)	☐	☐	
Other	☐	☐	

2. Does the child have any pre-existing chronic diseases (If yes, tick all that apply)? Yes ☐ No ☐

Diabetes ☐ Cerebral palsy ☐ Congenital abnormalities ☐ Heart disease ☐ Asthma ☐

Other ☐ specify _____

Complete Q5a) and 5b) for children ≥ 10 years only

5 a) Tanner scales (1= pre-pubescent; 5=adult) N/A ☐ Female Breasts ☐ Male Genitalia ☐ Pubic Hair ☐

b) Has the girl started menstruating? Yes ☐ No ☐ N/A ☐ If yes, date of menarche: M M M Y Y Y Y

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7. **What was the child's birth weight?** ☐☐ • ☐ kgs Not known ☐

8. **Was the child born premature (before 36 weeks gestation)?** Yes ☐ No ☐ Not known ☐
If yes, what was their gestational age? ☐☐ weeks Not known ☐

MEDICATION

13. **Is the child currently taking any oral/IV medication, including the contraceptive pill?** Yes ☐ No ☐
If yes provide details below. Exclude: paracetamol, cough and cold remedies, vitamin supplements, and any creams. Continue on a separate sheet if needed.

Drug Name (Name and Code)	Reason prescribed (e.g. contraception, or disease such as TB)	Daily dose	Date prescribed	No. Days prescribed
			D D M M M 2 0 1 Y	
			D D M M M 2 0 1 Y	
			D D M M M 2 0 1 Y	
			D D M M M 2 0 1 Y	
			D D M M M 2 0 1 Y	
			D D M M M 2 0 1 Y	
			D D M M M 2 0 1 Y	

14. **Is the child currently receiving any nutritional supplement?** ..... Yes ☐ No ☐
If Yes (tick all that apply)
Cereal ☐ Milk powder ☐ Plumpynut ☐ Soya flour ☐ Iron ☐
Folic acid ☐ Multivitamins ☐ Zinc ☐ Other ☐

INVESTIGATIONS and PLASMA/CELL STORAGE

19. **Investigations required**
a) **Has blood been taken for immunophenotyping, haematology, lipids?** *(complete Forms 11-13)* Yes ☐
b) **Has plasma been collected for storage?** *(complete Form 14)*..... Yes ☐ No ☐
c) **Has 2ml of citrated blood been taken for cardiovascular substudy?** *(complete Form 14)* ..... Yes ☐ No ☐

FORM 4 – LIPODYSTROPHY AND SOLICITED AEs

1. **Have either of the child's biological parents or grandparents suffered from the following conditions?**

Condition	Biological Mother			Biological Father			Grandparents			If yes, specify which grandparent:			
	No	Yes	Not known	No	Yes	Not known	No	Yes	Not known	PGM	PGF	MGM	MGF
Diabetes	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Hypertension	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Stroke	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Coronary artery disease	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Diagnosed with/treated for high cholesterol/triglycerides	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

MEDICAL HISTORY

2. Do either of the child's biological parents currently have lipodystrophy (fat wasting or accumulation)?

	Biological Mother			Biological Father		
	No	Yes	Not known	No	Yes	Not known
Fat accumulation	☐	☐	☐	☐	☐	☐
Fat wasting	☐	☐	☐	☐	☐	☐

BODY CIRCUMFERENCES AND SKINFOLD THICKNESSES

Not done ■

Section 3 + 4 completed by	Signature

3. Body circumferences (Should be left side where possible)

		1:		2:		3:	
Mid-upper arm	L / R*	.	cms	.	cms	.	cms
Waist		.	cms	.	cms	.	cms
Hip		.	cms	.	cms	.	cms
Mid-thigh	L / R*	.	cms	.	cms	.	cms
Calf	L / R*	.	cms	.	cms	.	cms

* circle as appropriate

4. Skinfold thicknesses (Should be left side where possible)

		1:		2:		3.	
Biceps	L / R*		mms		mms		mms
Triceps	L / R*		mms		mms		mms
Subscapular	L / R*		mms		mms		mms
Supra-iliac	L / R*		mms		mms		mms
Mid-thigh	L / R*		mms		mms		mms

* circle as appropriate

PHYSICIAN – other signs/symptoms and solicited adverse events

8. Do you consider that the child has:

Fat wasting	No ☐	Yes ☐	If yes, grade	☐
Fat accumulation	No ☐	Yes ☐	If yes, grade	☐
Peripheral neuropathy	No ☐	Yes ☐	If yes, grade	☐
Ascites	No ☐	Yes ☐		
Clinical malnutrition	No ☐	Yes ☐		

9. Does the child have trouble with:

Grading:
1 = Mild, not affecting day to day activities
2 = Affecting day to day activities but functioning near normal; not considering stopping medication.
3 = Severely affecting day to day activities; unable to attend school, considering stopping medication.

Concentrating at school/home	No ☐	Yes ☐	If yes, grade	☐
Vivid colourful dreams/nightmares	No ☐	Yes ☐	If yes, grade	☐
Sleepiness/Sleepwalking	No ☐	Yes ☐	If yes, grade	☐
Difficulty waking up in the morning	No ☐	Yes ☐	If yes, grade	☐
Waking up at night	No ☐	Yes ☐	If yes, grade	☐
Dizziness	No ☐	Yes ☐	If yes, grade	☐

COMMENTS

FORM 17 – SOCIOECONOMIC QUESTIONNAIRE

1. Which ethnic group or tribe does the child's mother align to? (tick one)

☐ Chewa	☐ Tumbuka	☐ Lomwe	☐ Yao	☐ Iteso	☐ Langi	☐ Acholi
☐ Alur	☐ Muganda	☐ Munyankole	☐ Munyarwanda	☐ Musoga	☐ Mukiga	☐ Sena
☐ Ngonde	☐ Mugisu	☐ Lugbara	☐ Munyoro	☐ Tonga	☐ Bemba	☐ Lozi
☐ Lunda	☐ Luvale	☐ Nyanja	☐ Kaonde	☐ Undisclosed	☐ Other, specify: _____	

2. Which ethnic group or tribe does the child's father align to? (tick one)

☐ Chewa	☐ Tumbuka	☐ Lomwe	☐ Yao	☐ Iteso	☐ Langi	☐ Acholi
☐ Muganda	☐ Munyankole	☐ Munyarwanda	☐ Alur	☐ Musoga	☐ Mukiga	☐ Sena
☐ Ngonde	☐ Mugisu	☐ Lugbara	☐ Munyoro	☐ Tonga	☐ Bemba	☐ Lozi
☐ Lunda	☐ Luvale	☐ Nyanja	☐ Kaonde	☐ Undisclosed	☐ Other, specify:	

THE HOUSE WHERE THE CHILD LIVES

3. Specify the type of house the child lives in? (tick all that apply)

a) Roof material:	☐ Grass thatched	☐ Corrugated sheets	☐ Asbestos	☐ Plastic	☐ Tiles
b) Building material:	☐ Mud/thatch/clay	☐ Sun-dried bricks	☐ Burnt bricks	☐ Stone	☐ Cement
c) Floor Materials:	☐ Earth/sand	☐ Wood/planks	☐ Palm/bamboo	☐ Broken bricks	☐ Cement ☐ Tiles
d) Tenure:	☐ Own (built)	☐ Own (bought)	☐ Renting	☐ Keeping for/living with relatives	

4. a) How many habitable rooms are in the house? ☐☐ **b) Is kitchen a separate room?** Yes ☐ No ☐

5. What type of toilet does the house have? ☐ Flush ☐ Pit/Latrine ☐ Other, specify _____

6. Does the house have electricity (mains or generator)? ..... Yes ☐ No ☐

7. What's the main fuel for cooking? ☐ Wood/charcoal ☐ Electric ☐ Gas ☐ Other, specify _____

8. Where does the household get water from (tick all that apply)?

☐ Protected well ☐ Domestic Tap ☐ Communal Tap ☐ Lake/river/stream

☐ Unprotected well ☐ Other (specify): _____

9. Which of the following items are found in the house (tick all that apply)?

☐ Fridge ☐ Clock ☐ Radio ☐ TV ☐ Video ☐ Sewing machine

☐ Telephone (landline) ☐ Telephone (mobile) ☐ Motor Bike ☐ Bicycle ☐ Car

10. How many adults (16 years or more) live in the same household as the child?

11. How many other children (0-15 years) live in the same household as the child? ☐☐

SCHOOL, EMPLOYMENT AND EXPENDITURE IN THE HOUSEHOLD

12. Does the child who is in clinic today go to school? ..... Yes ☐ No ☐

a) If yes, how many days of school, in the past 4 weeks, has s/he missed because of being sick?

(enter "00" if no days missed) ..... ☐☐

13. Does the child who is in clinic today go to work? ..... Yes ☐ No ☐

b) If yes, how many days of work, in the past 4 weeks, has s/he missed because of being sick?

(enter "00" if no days missed) ..... ☐☐

14. How many days employment for the adult household members has been lost in the last 4 weeks through illness of the child in clinic today? ..... ☐☐

15. What is the level of education attainment of the primary carer?

☐ No education ☐ Primary (uncomplete) ☐ Primary (complete) ☐ Secondary (complete) ☐ Tertiary (complete)

16. What is the source of income of the PRIMARY CARER? (tick all that apply)

Technician/artisan	☐	Student	☐	Market related jobs	☐	Driver/conductor/vehicle broker	☐
Public servant	☐	Farmer	☐	Soldier/policeman	☐	Bar or restaurant attendant	☐
Domestic worker	☐	Fishing	☐	Building/labouring	☐	Office worker	☐
Other	☐	specify:	_____				

17. What is the primary carer's current employment status? (tick one box)

☐ Working full time ☐ Working part time ☐ Working occasionally ☐ Full time student

☐ Not working due to ill health ☐ Not working due to lack of employment

22. Is there enough food to provide everyone in their household with regular meals? Yes ☐ No ☐

23. Does anyone in their household grow his/her own crops? Yes ☐ No ☐

FORM 19 – CARDIOVASCULAR SUBSTUDY

1. Please complete the following date and time details:

(a) Last meal: D D M M M 2 0 1 Y time (24:00): h h : m m Estimate ☐

(b) Time of scans time (24:00): h h : m m

CAROTID INTIMAL MEDIAL THICKNESS — Not done ■

Scan completed by	Signature

2. BP Cuff size? Small ☐ Large ☐

3. Right CCA (a) Number of recordings ☐☐

Comments: _____

_____

(b) Right arm BP (3 readings one minute apart):

	1:	2:	3:
Systolic	☐☐☐ mmHg	☐☐☐ mmHg	☐☐☐ mmHg
Diastolic	☐☐☐ mmHg	☐☐☐ mmHg	☐☐☐ mmHg
HR	☐☐☐ bpm	☐☐☐ bpm	☐☐☐ bpm

4. Left CCA (a) Number of recordings ☐☐

Comments: _____

_____

(b) Left arm BP (3 readings one minute apart):

	1:	2:	3:
Systolic	☐☐☐ mmHg	☐☐☐ mmHg	☐☐☐ mmHg
Diastolic	☐☐☐ mmHg	☐☐☐ mmHg	☐☐☐ mmHg
HR	☐☐☐ bpm	☐☐☐ bpm	☐☐☐ bpm

PULSE WAVE VELOCITY — Not done ■

Scan completed by	Signature

5. Room Temperature ☐☐ • ☐ °C

6. Side used R / L (should be RIGHT if possible) Right ☐ Left ☐

7. Leg cuff used SC5 ☐ TMC7 ☐ SC10 ☐

8. Details of PWV Carotid-Femoral measurements to nearest millimetre (MUST BE ACCURATE):

(a) Distance from suprasternal notch to base of carotid cuff: ☐☐ • ☐ Cms

(b) Distance from suprasternal notch to middle of thigh cuff: ☐☐ • ☐ Cms

(c) Direct distance from base of carotid cuff to middle of thigh cuff ☐☐ • ☐ Cms

IF THE CUFFS NEED REPOSITIONING AT ANY POINT PLEASE REMEASURE ALL DISTANCES AND DOCUMENT BELOW

(d) Distance from suprasternal notch to base of carotid cuff: ☐☐ • ☐ cms N/A ☐

(e) Distance from suprasternal notch to middle of thigh cuff: ☐☐ • ☐ cms N/A ☐

(f) Direct distance from base of carotid cuff to middle of thigh cuff ☐☐ • ☐ cms N/A ☐

9. How many PWV measurements have been taken? ☐☐

10. Please record details below. If more than 3 measurements taken, please document those for analysis (3 readings all within 0.5m/s).

	PWV		For analysis?	
1:	☐☐•☐	m/s	Yes ☐	No ☐
2:	☐☐•☐	m/s	Yes ☐	No ☐
3:	☐☐•☐	m/s	Yes ☐	No ☐
4:	☐☐•☐	m/s	Yes ☐	No ☐
5:	☐☐•☐	m/s	Yes ☐	No ☐
6:	☐☐•☐	m/s	Yes ☐	No ☐

Comments : _____

_____

Has form 7 Lab request - controls been completed.....Yes ☐ No ☐
Has form 11 Haematology - controls been completed.....Yes ☐ No ☐
Has form 12 Biochemistry - controls been completed.....Yes ☐ No ☐
Has form 13 Lymphocyte subsets - controls been completed.....Yes ☐ No ☐
Has form 14 Sample Storage- controls been completed .....Yes ☐ No ☐

Form completed by	Print name	Date
		D D M M M 2 0 1 Y

CHAPAS-3

CHAPAS-3 TRIAL **FORM 7 – LAB REQUEST - CONTROLS**

Trial Number | Male ○ Female ○ | Date of Birth D D M M M Y Y Y Y

Visit Date D D M M M 2 0 1 Y | Child's Initials | ART/clinic number

This visit is week number: Screening

TRIAL SCHEDULED INVESTIGATIONS

Request for

Confirmatory HIV-1 test	Yes ☐	No ☐	(if yes, fill Form 10)
Haematology	Yes ☐	No ☐	(if yes, fill Form 11)
Biochemistry	Yes ☐	No ☐	(if yes, fill Form 12)
Lymphocyte subsets.....	Yes ☐	No ☐	(if yes, fill Form 13)
Sample storage	Yes ☐	No ☐	(if yes, fill Form 14)

IMMUNOLOGY SUBSTUDY INVESTIGATIONS & STORAGE

Request for

Immunophenotyping..... Yes ☐ No ☐ (if yes, fill Form 13)

(Using blood already taken for lymphocyte subsets/haematology

SAMPLE IDENTIFIER

Nurse or Doctor's signature	Print name	Date
		D D M M M 2 0 1 Y

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CHAPAS-3

CHAPAS-3 TRIAL

FORM 11 – HAEMATOLOGY - CONTROLS

Trial Number

Male ○ Female ○

Date of Birth D D M M M Y Y Y Y

Visit Date D D M M M 2 0 1 Y

Child's Initials

ART/clinic number

COMPLETE REQUEST AT CLINIC (by ticking boxes) and INCLUDE with SPECIMENS

ENTER RESULTS AT LABORATORY (in grey areas/attach to form) and RETURN to CHAPAS-3 Clinic

Week number (If unscheduled, tick extra)

Extra ☐

Sample Identifier

☐ Trial Scheduled Haematology

Date of test: D D M M M 2 0 1 Y

		Not Done
Haemoglobin	. g/dl	☐
MCV	. fl	☐
White cell count	. $x10^3$ cells /mm^3	☐
Neutrophils	. $x10^3$ cells /mm^3	☐
Lymphocytes	. $x10^3$ cells /mm^3	☐
Platelets	$x10^3$ cells /mm^3	☐

Comments: _____

Clinic Nurse or Doctor's signature	Print name	Date
		D D M M M 2 0 1 Y

Laboratory personnel's signature	Print name	Date
		D D M M M 2 0 1 Y

ISRCTN69078957

CHAPAS-3

CHAPAS-3 TRIAL **FORM 12 – BIOCHEMISTRY - CONTROLS**

Trial Number | Male ○ Female ○ | Date of Birth D D M M M Y Y Y Y

Visit Date D D M M M 2 0 1 Y | Child's Initials | ART/clinic number

COMPLETE REQUEST AT CLINIC (by ticking boxes) and INCLUDE with SPECIMENS

ENTER RESULTS AT LABORATORY (in grey areas/attach to form) and RETURN to CHAPAS-3 clinic

Week number (If unscheduled, tick extra) | Extra ☐ | Sample Identifier

☐ **Additional tests** (tick and specify tests required. TC, LDL, HDL, Triglycerides to be requested at enrolment, week 48 and week 96)

Date of test D D M M M 2 0 1 Y

☐ ALK U/l

☐ Total Cholesterol . mmol/l mg/dl (delete as appropriate)

☐ LDL . mmol/l mg/dl (delete as appropriate)

☐ HDL . mmol/l mg/dl (delete as appropriate)

☐ Triglycerides . mmol/l mg/dl (delete as appropriate)

☐ _____ . Specify units: _____

☐ _____ . Specify units: _____

☐ _____ . Specify units: _____

☐ _____ . Specify units: _____

Comments: _____

Clinic Nurse or Doctor's signature	Print name	Date
		D D M M M 2 0 1 Y

Laboratory personnel's signature	Print name	Date
		D D M M M 2 0 1 Y

CHAPAS-3 TRIAL

FORM 13 – LYMPHOCYTE SUBSETS - CONTROLS

Trial Number | Male ○ Female ○ | Date of Birth D D M M M Y Y Y Y

Visit Date D D M M M 2 0 1 Y | Child's Initials | ART/clinic number

COMPLETE REQUEST AT CLINIC (by ticking box) and INCLUDE with SPECIMENS
ENTER RESULTS AT LABORATORY (in grey areas/attach to form) and RETURN to Clinic

Week number (If unscheduled, tick extra) | Extra ☐ | Sample Identifier

☐ Scheduled Lymphocyte Subsets

Date of test D D M M M 2 0 1 Y

	Percentage		Count	
CD3	. %			cells /mm^3 or /µl
CD4	. %			cells /mm^3 or /µl
CD8	. %			cells /mm^3 or /µl
Total Lymphocytes	. %			cells /mm^3 or /µl
Total WBC			.	cells /mm^3 or /µl

☐ Immunology Substudy Lymphocyte Subsets (immunophenotyping assays requested at same time as scheduled lymphocyte subsets at JCRC and UTH).

Date of test D D M M M 2 0 1 Y

PANEL 1: CD38 AND HLA-DR — Not done ■

T cell subset	% of CD3+ CD8- T lymphocyte (% GATED) Quadrant		T cell subset	% of CD3+ CD8+ T lymphocyte (% GATED) Quadrant	
CD3+CD8-CD38+	UL+UR	. %	CD3+CD8+CD38+	UL+UR	. %
CD3+CD8-HLADR+	UR+LR	. %	CD3+CD8+HLADR+	UR+LR	. %
CD3+CD8-CD38+HLADR-	UL	. %	CD3+CD8+CD38+HLADR-	UL	. %
CD3+CD8-CD38+HLADR+	UR	. %	CD3+CD8+CD38+HLADR+	UR	. %
CD3+CD8-CD38-HLADR-	LL	. %	CD3+CD8+CD38-HLADR-	LL	. %
CD3+CD8-CD38-HLADR+	LR	. %	CD3+CD8+CD38-HLADR+	LR	. %

PANEL 2: CD31 AND Ki67 — Not done ■

T cell subset	Quadrant*	% of CD4+ T lymphocyte (% GATED)	T cell subset	Histogram*	% expression of Ki67 (% GATED M1)
CD4+CD45RA-CD31+	UL	. %	CD4+ T cells	G3	. %
CD4+CD45RA+CD31+	UR	. %	CD4+CD45RA-CD31+	G4	. %
CD4+CD45RA-CD31-	LL	. %	CD4+CD45RA+CD31+	G5	. %
CD4+CD45RA+CD31-	LR	. %	CD4+CD45RA-CD31-	G6	. %
			CD4+CD45RA+CD31-	G7	. %

*** Please note that quadrants above are listed in a different order to those on the ARROW form**

***Histograms above are labelled differently to ARROW**

Clinic Nurse or Doctor's signature	Print name	Date
		D D M M M 2 0 1 Y

Laboratory personnel's signature	Print name	Date
		D D M M M 2 0 1 Y

ISRCTN69078957

CHAPAS-3

CHAPAS-3 TRIAL **FORM 14 – SAMPLE STORAGE - CONTROLS**

Trial Number		Male ○ Female○	Date of Birth D D M M M Y Y Y Y
Sample Date D D M M M 2 0 1 Y		Child's Initials	ART/clinic number

COMPLETE REQUEST AT CLINIC (by ticking box) and INCLUDE with SPECIMENS.
ENTER RESULTS AT LABORATORY (in grey areas) and RETURN to Clinic

To be filled out by doctor or nurse requesting the storage: Week number

☐ Routine EDTA plasma storage – please store in CONTROL cryobox

☐ Cell pellet storage

☐ CV cryobox: 1 X citrated plasma & 1 X EDTA plasma

Clinic Nurse or Doctor's signature	Print name	Date D D M M M 2 0 1 Y

To be filled out by laboratory staff:

Sample Identifier		Date of storage D D M M M 2 0 1 Y

1. Has one or more 1ml aliquots of **routine EDTA plasma** been stored (with patient identification labels)? Yes ☐ No ☐
2. Has one or more **DNA cell pellets** been stored (with patient identification labels)? Yes ☐ No ☐
5. Has **citrated plasma and 1 x EDTA plasma** been stored in CV cryobox? Yes ☐ No ☐
6. Has **whole blood** been stored? Yes ☐ No ☐

If yes to any, complete table below for each stored aliquot/pellet. Whenever possible a minimum of **3 routine plasma aliquots (but no aliquot to have volume less than 500µl)** should be stored and a minimum of 1 cell pellet.

	Volume of Sample Stored (Specify Units)	Rack	Tray/Box number	Position	Aliquot/pellet ID Number
EDTA plasma aliquot #1					
EDTA plasma aliquot #2					
EDTA plasma aliquot #3					
EDTA plasma aliquot #4					
DNA pellet #1					
DNA pellet #2					
DNA pellet #3					
Citrated plasma #1					
Citrated plasma #2					
Whole Blood					

Laboratory personnel's signature	Print name	Date D D M M M 2 0 1 Y

ISRCTN69078957

Appendix II: Presentations, posters, published abstracts and papers

a) Kenny J, Mirembe G, Musiime V, Masaku D, Kavindele D, Odongo F, Wavamunno P, Rapala A, Cook A, Deanfield J, Gibb D, Klein N. Cardiovascular structure and function in HIV-infected children in Zambia and Uganda. *Reviews in Antiviral Therapy & Infectious Diseases.* 2012 (8) Oral abstract 17, page 19.
   - Oral presentation at the 4th International Pediatric HIV Workshop, Washington, July 2012

b) Kenny J, Mirembe G, Musiime V, Masaku D, Odongo F, Wavamunno P, Thomason M, Rapala A, Cook A, Deanfield J, Walker S, Gibb D, Klein N. Structural cardiovascular changes are reversible in HIV-infected children in Zambia and Uganda
   - Oral presentation at CROI 2015, Seattle USA.

c) Kenny J, Walker S, Cook A, Musiime V, Wavamunno P, Odongo F, Mirembe G, Masaku D, Gibb D, Klein N. The impact of HIV and ART on markers of inflammation, vascular injury and disordered thrombogenesis in children
   - Poster presentation at CROI 2015, Seattle USA.

d) Kenny J, Mulenga V, Hoskins S, Sholten F, Gibb D. The needs for HIV treatment and care of children, adolescents, pregnant women and older people in low and middle-income countries. *AIDS*. 2012 Dec (26) Suppl 2:S105-16.

e) Kenny J, Musiime V, Judd A, Gibb D. Recent advances in pharmacovigilance of antiretroviral therapy in HIV-infected and exposed children. *Current Opinion in HIV/AIDS.* 2012 7 (4) 305–316.

Oral abstract presentation at the 4th International Pediatric HIV Workshop, Washington, July 2012

Kenny J, Mirembe G, Musiime V, Masaku D, Kavindele D, Odongo F, Wavamunno P, Rapala A, Cook A, Deanfield J, Gibb D, Klein N. **Cardiovascular structure and function in HIV-infected children in Zambia and Uganda** Reviews in Antiviral Therapy & Infectious Diseases 2012 8 Oral abstract 17, page 19.

[1]Institute of Child Health, University College London, [2]Clinical Trials Unit, Medical Research Council, [3]Joint Clinical Research Centre, Lubowa, Kampala, [4]University Teaching Hospital, Lusaka, [5]Vascular Physiology Laboratory, Institute of Child Health, University College London.

Background / introduction

Carotid intimal medial thickness (cIMT) and pulse wave velocity (PWV), as measures of cardiovascular structure/function, are impaired in HIV-infected UK children, although the impact of ART and obesity/lifestyle factors has been difficult to determine. No studies have been undertaken in Africa where 90% HIV-infected children live. We are undertaking a longitudinal study of arterial structure/function, nested within a large randomised trial (CHAPAS 3) of different ART regimens in Uganda/Zambia. We present baseline data on ART-naïve and ART-experienced children.

Methods

All cooperative ART-naive and experienced (stable on d4T+3TC+NNRTI for >2years) HIV-infected children recruited from 2 sites in Uganda/Zambia had baseline cIMT and PWV measurements undertaken by the same operators in each country. Differences between ART-naïve and experienced children, accounting for age and ART duration, were analysed using linear regression.

Results

180(121:59 ART-naive:experienced) children had cIMT and 212(139:73) had PWV measured; median(range) age was 4.7(0.7-13.6 years); 53% male; median CD4% in naïve:experienced 18:33%.

In ART-naïve vs experienced children, mean(sd) cIMT was 0.46(0.44)mm vs 0.47(0.35)mm (p=0.4) and PWV was 5.79(0.8)metre/second vs 5.63(0.61)metre/second (p=0.14), with a non-significant trend towards IMT and PWV increasing with age. (IMT p=0.08, PWV p=0.09) In ART-experienced children, duration of ART (median(range) 3.9(2-6.2 years)) did not influence cIMT(p=0.2) or PWV(p=0.5). Values were comparable to those of UK HIV-uninfected and lower than HIV-infected children.

Discussion

In this first large study of arterial structural and function in HIV-infected children in Africa, results were similar to HIV-uninfected UK children and were unrelated to ART status. Follow-up data, comparison with African controls, and further analyses exploring the impact of lipid levels and markers of inflammation on these vascular markers may all provide insight into the pathogenesis of HIV and ART-mediated mechanisms of vascular injury and repair as well as providing guidance on optimal first-line ART for HIV-infected children in Africa.

CROI 2015, Seattle, USA. Oral presentation.

Kenny J, Mirembe G, Musiime V, Masaku D, Odongo F, Wavamunno P, Thomason M, Rapala A, Cook A, Deanfield J, Walker S, Gibb D, Klein N.

Structural cardiovascular changes are reversible in HIV-infected children in Zambia and Uganda

Background

Carotid intimal medial thickness (IMT) and pulse wave velocity (PWV), as measures of cardiovascular structure/function, are impaired in HIV-infected children in high-income countries. Few longitudinal data are available: none come from Africa where 90% HIV-infected children live.

Methods

ART-naïve and ART-experienced (on d4T+3TC+NNRTI for >2years, virologically suppressed at enrolment) HIV-infected children had IMT and PWV measured at baseline, 48 and 96 weeks within the CHAPAS-3 trial which evaluated d4T vs ZDV vs ABC-based first-line ART in Uganda/Zambia. Age-matched HIV-uninfected controls had a single assessment. Baseline differences between ART-naïve/experienced children vs controls, and longitudinal changes in HIV-infected children were compared using two-sample and paired t-tests respectively.

Results

In 208 ART-naïve children with median age 2.9y (IQR 1.7–4.4), median CD4% 18% (11-23) and 209 HIV-uninfected controls median age 3.0y (2.1–4.1), mean(sd) cIMT was 0.46(0.04) v 0.44(0.04)mm respectively (p=0.0001); PWV was 5.85(0.8) vs 5.67(0.74)m/sec respectively (p=0.04). Among 74 ART-experienced children on ART for mean 3.7y with median age 6.9y (5.9–8.50, median CD4% 33% (27-39) and 75 uninfected controls with median age 6.7y (5.6-8.6), mean(sd) cIMT was 0.46(0.05) vs 0.45(0.04)mm respectively (p=0.09); PWV was 5.63(0.61) vs 5.69(0.69)m/s respectively (p=0.57). In ART naïve children IMT and PWV significantly decreased from baseline (ART initiation) to week 96 mean(sd) cIMT -0.02(0.04)mm (p=0.0001), PWV -0.38(0.83)m/s (p<0.0001). In contrast whereas cIMT had significantly reduced by mean -0.2(0.06)mm (p=0.01) at week 96 in the ART experienced group PWV increased by 0.35(0.63)m/s (p<0.0001). There was no evidence that the changes differed by randomisation ART in either group (p=0.6).

Conclusion

In this large study of arterial structural and function in HIV-infected children in Africa, ART-naïve HIV-infected children had significantly poorer IMT and PWV compared to age-matched controls but significant improvement seen after 96 weeks of ART. After a mean 3.7 years on ART, HIV-infected children had cIMT and PWV comparable to uninfected age-matched controls. IMT continued to improve after a further 96 weeks on ART. ART can reverse some of the structural/functional changes caused by HIV, strengthening the argument for early diagnosis and treatment of HIV-infected infants and children.